Dietary Lysine and Arginine Requirements of Juvenile and Adult Zebrafish (*Danio rerio*)

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ABSTRACT

Use of zebrafish as a model organism in the biomedical field has increased dramatically in the past 20 years. Optimal culture conditions such as water temperature and water chemistry values have been established for zebrafish, while the development of a standardized diet has lagged behind. Failure to control dietary variables may influence experimental outcomes. In order for a standardized diet to be adequate, the feed must meet or exceed the zebrafish's macronutrient requirements. To date, there has been no published data on quantitative macronutrient requirements of zebrafish. Two dose-response experiments utilizing isonitrogenous and isocaloric diets were performed in order to determine the dietary lysine and arginine requirements for zebrafish. Each experiment was divided into a juvenile and adult study. The dietary lysine requirement for juvenile and adult zebrafish was found to be 2.2% of dry diet and 5.6% of protein. The dietary arginine requirement for juvenile zebrafish was unable to be determined while the requirement for adult zebrafish was found to be 1.95% of dry diet and 6.5% of protein. Lysine-arginine antagonism was not observed in zebrafish. Plasma free arginine concentrations remained relatively stable in zebrafish fed diets ranging from 0.80 to 2.70% arginine. Plasma free proline decreased with decreasing dietary arginine, suggesting proline is being scavenged to synthesize arginine. In the future, adjustments should be made to experimental diet formulation with the goal of achieving growth performance similar to that of a reference diet.

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LITERATURE REVIEW

ZEBRAFISH IN NATURE

Zebrafish (*Danio rerio*) are small (~30 mm standard length) freshwater fish belonging to the Cyprinid (minnow) family. They have a terminal mouth at the tip of their fusiform body, which is laterally compressed, typical of most minnow species. The common name "zebrafish" comes from their distinctive light and dark longitudinal stripes that run from the operculum to the caudal fin.

The native territory of zebrafish is the Ganges and Brahmaputra River basins located in India, Bangladesh, and Nepal (Laale, 1977; Barmann, 1991). They are a naturally shoaling species that typically inhabit warmwater rivers and streams with shallow, clear-water and a variety of substrata (McClure et al., 2006). It has been suggested that zebrafish are actually more of a flood-plain species than a true riverine species and are often associated with waters connected to rice cultivation (Spence et al., 2006). "Danio" comes from the word "dhani" which means "of the rice field" (Talwar and Jhingaran, 1991). Zebrafish have been found in various habitats; occupying the entire water column and can be found in open water as well as among aquatic vegetation (Spence et al., 2006). Variation in habitat makes them susceptible to many predators including snakeheads, freshwater garfish, common kingfisher, and Indian pond heron (Spence et al., 2008). Zebrafish are also susceptible to their own species, as they will predate their own eggs and larvae.

Zebrafish are considered to be an omnivorous species, eating a variety of foods. Insects are the most abundant food source in the zebrafish diet, which can be of aquatic or terrestrial origin (McClure et al., 2006; Spence et al., 2007). Filamentous algae and

plant material have also been found in the zebrafish gut (Spence et al., 2007). These food sources can come from many places as zebrafish have been observed feeding in the water column, on the surface and on the bottom (Spence et al., 2007).

Male and female zebrafish are difficult to tell apart as there are no distinguishing external characteristics to differentiate them. In general females tend to be more rounded while males generally have a streamlined appearance and a larger anal fin with a yellowish hue. Zebrafish are a predominantly annual species that breeds primarily during the monsoon season when food is plentiful (Spence et al., 2006). However, they are considered continuous spawners when food is available throughout the year (Spence et al., 2006).

ZEBRAFISH IN THE LABORATORY

The aquarium-fish community has long known about zebrafish, often called "Zebra Danio", as they are a popular aquarium fish that can be found throughout the world. Their hardiness and low-cost have made them popular for novice aquarists. These same characteristics have made them an attractive model organism starting in 1951 when the first article on zebrafish was published (Kinth et al., 2013). Many biological characteristics unique to zebrafish have contributed to their popularity as a model organism such as their high fecundity, short generation time, predictable spawning time and low cost of maintenance. In general they are a hardy species and can withstand a wide range of culture conditions whether it be temperature, salinity, dissolved oxygen, pH or nitrates (Lawrence, 2007). Not only can zebrafish withstand wide ranges of these water quality parameters but they can also handle abrupt changes in them as well. This

"forgiving" characteristic of zebrafish enables those who are not well adept at fish husbandry the ability to care for them and establish a healthy reproductive colony.

Approximately 70% of the human genes have a homolog in the zebrafish genome (Langheinrich, 2003). Tools have been developed and refined so that researchers can quickly and easily make zebrafish transgenic and mutant lines, which makes it a viable model for human genetics research. Along with genetics, zebrafish are also a popular model organism for use in the fields of toxicology, developmental biology, pharmacology, neurology, biochemistry and cell biology (Kinth et al., 2013). Zebrafish embryos and larvae are transparent, thus in combination with a transgenic fluorescent protein promoter, make it easy for researchers to conduct image analysis of whatever gene or transcription factor they are studying. The popularity of zebrafish as a model organism has steadily grown since the 1990's when the zebrafish genome was first mapped. Today they are cultured in many biomedical research facilities around the world including the United States (877 institutions), Germany (359), England (180), China (255), France (219), Spain (138), Taiwan (84), Singapore (34), Australia (74), Israel (30), Brazil (51), Chile (16), and Portugal (39) (Kinth et al., 2013). Estimating the exact numbers of zebrafish used for research is almost impossible, however it is believed that millions, if not hundreds of millions are used for scientific research every year. Zebrafish are now considered one of the most popular model organisms, surpassing *Drosophila* (Kent and Varga, 2012), and are often termed the "aquatic lab rat".

Spawning

Zebrafish are known for their plentiful egg production. Similar to most fish, zebrafish eggs are fertilized externally, which requires a courtship ritual to stimulate the release of the female's eggs (Spence et al., 2008). Females are unable to release their eggs unless they are exposed to male gonadal pheromones (Van den Hurk and Lambert, 1983, Van den Hurk et al., 1987). Courtship often consists of abrupt turns in an elliptical pattern by the male around the female (Guthrie, 1986). Three phases of courtship have been identified: initiatory, receptive/appetitive and spawning (Darrow and Harris, 2004). Zebrafish have been shown to recognize their kin via olfactory cues and thus avoid spawning with related individuals (Gerlach and Lysiak, 2006). During the act of spawning females release their eggs which slowly sink to the bottom of the tank or substrate while males simultaneously release their sperm which fertilizes the eggs. Fertilized eggs are non-adhesive and are approximately 0.7mm in diameter. Unlike other fish, fertilized embryos of zebrafish do not require parental care, hatch in about 3 to 5 days and are considered adults at 90 days post fertilization (dpf) (Gomez-Requeni et al., 2010).

Female zebrafish have been known to lay up to 1800 eggs in a spawning session, but production is often between 150 to 400 eggs (Laale, 1977). Zebrafish are considered continuous spawners meaning that they will spawn continuously throughout their life without seasonal cues (Spence et al., 2006). It is typical of laboratory zebrafish to spawn every week or every-other week for over two years. This process is beneficial to researchers who may need a large amount of embryos in a short period of time.

Husbandry

Housing of zebrafish is relatively inexpensive (Spence et al., 2008; Santoriello and Zon, 2012) because they are a naturally shoaling species so a large population can be housed in small tanks. Stocking densities vary from facility to facility but zebrafish can be housed at rates up to 15 adults L^{-1} without drastically altering water chemistry. However, zebrafish housed at densities of 40 fish L^{-1} displayed an increase in plasma cortisol levels (Ramsay et al., 2006). It is apparent that many zebrafish can be housed in a relatively small footprint, which has made them a popular model for smaller research facilities.

Optimal culture conditions such as water temperature and water chemistry values have been established for zebrafish, while the development of a standardized diet has lagged behind. Feeding practices vary greatly between laboratories and often include a combination of live feeds such as *Artemia* nauplii, paramecia, and rotifers; and feeding various uncontrolled or undefined commercial aquarium diets. Nutritional components and ingredients of these feeds are often unknown. Failing to control important variables such as diet may influence experimental outcomes (Lawrence, 2007; Siccardi et al., 2009; Kent and Varga, 2012; Penglase et al., 2012; Watts et al., 2012). The National Academy of Sciences states that animals used for experimental purposes should be fed a diet of detailed composition (Barnard et al., 2009). Many researchers in the biomedical field are now asking for a standardized open formula diet for zebrafish (Lawrence, 2007; Siccardi et al., 2009; Kaushik et al., 2011; Kent and Varga, 2012; Penglase et al., 2012; Watts et al., 2012).

Standardization of diets for model organisms is not a new concept in biomedical research. Demands for a standardized rodent diet occurred in the 1970's, which resulted in standard open formulation diets NIH07 and AIN76. Standardized diets for other model organisms soon followed including guinea pigs, rabbits, primates and swine. The lack of a standardized open formulated diet for zebrafish can lead to variability in research results, particularly when zebrafish are used for research in human disease, pharmacology, toxicology, gene expression, neurology and reproduction (Siccardi et al, 2009; Lawrence, 2011; Watts et al., 2012). Therefore, lack of a standardized diet has a drastic impact on the success of this important model organism (Watts et al., 2012). Until a standardized diet is employed by the zebrafish community, research results between different laboratories can not be fully trusted (Siccardi et al., 2009).

In order for a standardized diet to be adequate, the feed must meet or exceed the zebrafish's macronutrient requirements (Ahmed and Khan, 2004; Ulloa et al., 2011; Watts et al., 2012; Smith et al., 2013). Knowing the nutritional requirements of zebrafish is an important part in extending the utility of the zebrafish model (Robison et al., 2008) because different levels of carbohydrates and proteins as well as their sources can alter gene expression (Schwerte et al., 2005; Robison et al., 2008; Fuentes-Appelgren et al., 2014). Zebrafish are also thought to be a potential model organism for the aquaculture industry, which would enable research on dietary factors to proceed more quickly and cheaply, however this cannot be accomplished without first knowing the nutrient requirements of zebrafish (Gomez-Requeni et al., 2010; Fuents-Applegren et al., 2014).

Formulating a diet that does not meet the nutritional requirements of zebrafish could lead to poor health, growth and embryo production. Maintaining healthy,

reproductive zebrafish is not only crucial in terms of conducting research, but for meeting animal welfare standards set by the federal government. *The Guide for the Care and Use of Laboratory Animals* sets the standards for care and use of all laboratory animals, including aquatics, and states "care should be taken to feed a complete diet to avoid nutritional deficiencies" (Institute of Laboratory Animal Resources (US), 1985). Unfortunately, information concerning nutritional deficiencies in zebrafish is nonexistent.

Research on nutrition of zebrafish is in its infancy and is thought to be the least developed aspect of zebrafish husbandry (Lawrence, 2011; Kent and Varga, 2012). To date, there has been no published data on the macronutrient requirements of zebrafish. Nutrient requirements for other freshwater species have been determined, however requirements between fish species can vary considerably (Robison et al., 2008; National Research Council, 2011). This variability is often a result of their natural diet selection (carnivore, herbivore, detritivore, omnivore), native region, taxa and preferred water temperature. In general, it is thought that the macronutrient requirements of zebrafish will be similar to that of common carp (*Cyprinus carpio*) (Kaushik et al., 2011; Ulloa et al., 2011) due to their similar natural diet (omnivorous), taxa (*Cyprinidae* family), digestive tract (incomplete stomach) and natural habitat (warmwater). While no quantitative studies on nutrient requirements of zebrafish have been published, there has been some dietetics research that may provide some information on nutrient requirements of zebrafish.

FISH NUTRITION

Generalizing fish nutrition can be very difficult because there are approximately 30,000 species of fish that have been classified. Each species occupies its own feeding niche and thus has its own unique nutrient requirements. To put things in perspective, there are only ~5,500 species of mammals and it would be difficult to generalize mammal nutrition with diversity ranging from a blue whale (*Balaenoptera musculus*) that consume massive amounts of plankton, to a coyote (*Canis latrans*) who is strictly carnivorous, to a pocket gopher (*Geomys bursarius*) that consumes vegetative matter. Fish are no different, with diverse species feeding exclusively on algae, detritus or flesh. For generalization purposes this section will focus on freshwater omnivorous fish.

Similar to all animals, fish require energy, lipids, protein and amino acids, vitamins and minerals. However, the quantity and method of procurement of these macro and micronutrients differs from other animals.

Energy

Unlike mammals, fish are ectothermic so metabolic rate and energy expenditure is greatly affected by water temperature (National Research Council, 2011). Lipids and proteins are the primary energy sources for fish. Carbohydrates comprise a relatively small percentage of the fish diet. The usefulness of an energy source is greatly dependent upon its digestibility which differs in each particular species. Fecal energy losses often range from 15 to 30% of energy intake (National Research Council, 2011) but can be as much as 50 to 80% depending on the feedstuff (Bureau et al., 2002). Digestible energy (DE) values are preferred when estimating available energy levels of feed ingredients

(Cho and Kaushik, 1990). Metabolizable energy (ME) is not used because it is difficult to determine in fish. Detection of non-fecal energy losses requires restraining the fish in a sealed vessel and force-feeding the fish, which adds considerable stress (National Research Council, 2011). This method overestimates urinary and branchial losses that would normally be found in unrestrained fish that feed normally, resulting in lower ME estimates than actual ME (Cho and Kaushik, 1990). Nonfecal energy losses are often 3 to 6% of ME (Kaushik, 1998; Bureau et al., 2002), thus DE or gross energy values are commonly used energy measurements.

Lipids

Lipids are one of the two major energy sources for fish and provide essential fatty acids (EFA) via β-oxidation that are needed for normal growth and development. In general, basic pathways of lipid metabolism in fish are the same as in mammals (National Research Council, 2011). Digestibility of lipids is affected by fish species, lipid content and composition, water temperature and other dietary components (Hua and Bureau, 2009). Digestion of dietary lipids occurs primarily in the proximal intestine and pyloric caeca, resulting in mainly free fatty acids. The resulting free fatty acids are then transported in the blood by binding to albumin-like proteins or formed into various lipoproteins such as chylomicrons, high-density lipoproteins (HDL), low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL). Vitellogenin is a special lipoprotein that is produced by female fish to transport lipids to developing oocytes (National Research Council, 2011).

Excess dietary lipids are stored in various adipose tissues throughout a fish's body, however the location of the adipose tissue varies between species (National Research Council, 2011). While many species have intestinal adipose tissue, others deposit lipids in the liver, flesh or between the skin and flesh. It is worth noting that deposition of excess dietary lipid has been shown to influence sexual maturity in males (Shearer and Swanson, 2000).

Lipids provide energy and EFAs to fish. True lipid requirements for any species cannot be defined because it can be influenced by many nutritional factors (National Research Council, 2011). Proteins and carbohydrates are the primary carbon sources for lipogenesis and the quantity of these nutrients can influence lipid requirement. Essential fatty acid profiles differ greatly depending on lipid source, whether it is from various animal or plant sources, which also influence lipid requirement.

Contrary to what many believe, fish cannot synthesize polyunsaturated fatty acids (PUFA) from monounsaturated fatty acids, similar to all vertebrates. Most PUFA originate from microalgae that are then consumed by fish. A deficiency in these EFA in fish, more specifically n-3 and n-6 PUFA, can result in reduced growth and reproduction, and ultimately death (Das, 2006; Glencross 2009). PUFA with a double bond on the third carbon from the end are referred to as n-3 whereas PUFA with a double bond on the sixth carbon from the end are referred to as n-6. In terms of broodstock nutrition, EFA are important for producing quality embryos and larvae (Tandler et al., 1995; Izquierdo et al., 2001; Quintero et al., 2011). Other deficiency signs include fatty liver, intestinal steatosis, fin erosion, lordosis and reduced reproductive potential (Tacon, 1996). The shorter chain PUFA, linoleic acid 18:2n-6 and α-linolenic acid 18:3n-3 actually have no

specific metabolic role. However, most omnivorous fish are able to elongate these fatty acids (Meinelt et al., 2000) to n-3 and n-6 long chain PUFA 20:4n-6 (ARA, arachidonic acid), 20:5n-3 (EPA, eicosapentaenoic acid) and 22:6n-3 (DHA docosahexaegonoic acid) (Sargent et al., 1995) which are essential for several physiological processes (Das, 2006). Vertebrates are also incapable of converting n-6 to n-3 and vice versa, therefore both are required (National Research Council, 2011).

Quantitative EFA requirements have been determined for approximately 30 species of fish. Most freshwater species require n-3 and n-6 PUFA each at approximately 1% dry weight of the diet (National Research Council, 2011). Coldwater fish species often require a higher portion of n-3 than n-6, while warmwater fish require similar n-3 and n-6 quantities (Meinelt et al., 2000). Karanth et al. (2009) found that the proportion of fatty acids in the diet of zebrafish changed the fatty acid profile of their liver, intestine, muscle and brain. It has also been determined that the higher the content of n-6 fatty acids in the diet of adult zebrafish results in better growth and a higher fertilization rate (Meinelt et al., 1999; Meinelt et al., 2000). Meinelt et al. (2000) also suggested that the standardized diet for zebrafish should contain a low n-3:n-6 ratio in order to maximize growth and embryo production (Meinelt et al., 2000), while Jaya-Ram et al. (2008) proposed the need for a balanced ratio of n-3 and n-6 unsaturated fatty acids.

It has recently been found that PUFA influence regulation of gene transcription (Jump, 2002) by altering activities of several transcription factors including peroxisome proliferator activated receptors (PPAR) (Sampath and Ntambi, 2005). Influence of PUFA on gene transcription is of particular concern for the zebrafish community because

they are often used as a model organism for genetics research and the PUFA profiles of most diets that are used are not known or standardized.

Phospholipids are major components of cell membranes and lipoproteins. Inclusion of intact phospholipids in diets has been demonstrated to improve growth in larvae and juvenile fish (Tocher et al., 2008). While it is difficult to define absolute phospholipid requirements in adult fish, the requirement for larval fish is typically 8 to 12% of dietary dry matter (Cahu et al., 2009).

Cholesterol is essential for all animal cell function as it plays a role in intracellular transport, cell signaling and nerve conduction (National Research Council, 2011). Cholesterol is also a precursor for the synthesis of steroids and vitamin D_3. Zebrafish fed a high cholesterol diet (4% cholesterol) were found to exhibit hypercholesterolemia but did not show a difference in weight gain compared to the control (Stoletov et al., 2009). There are a few reports on effects of cholesterol on the growth of fish, however, there are currently no quantitative requirements for cholesterol in any species of fish (National Research Council, 2011).

Minerals

All animals require minerals to support normal life processes. Macrominerals such as calcium, chlorine, magnesium, phosphorus, potassium and sodium as well as microminerals such as chromium, copper, iodine, iron, manganese, selenium and zinc are required for fish. These minerals function in many areas such as skeletal tissue, membranes, osmotic balance, enzyme activation, acid-base equilibrium, carbohydrate metabolism and oxygen transport. Deficiencies in minerals can result in reduced growth,

hard tissue mineralization, skeletal deformities, fat accumulation, cataracts and anemia (National Research Council, 2011).

Fish are unique because they have the ability to obtain certain minerals from their external environment as well as from their diet. Depending on species and properties of the water in their environment, certain minerals may not be required in their diet. Quantifying mineral requirements is difficult due to ion exchange from the aquatic environment across the gills of freshwater fish.

Fishmeal is a rich source for many minerals and is commonly used when formulating practical fish diets (National Research Council, 2011). Plant based ingredients are often poor sources of minerals while bone meal, poultry byproduct meal and feather meal are rich in minerals. In general, supplemental minerals are often added to diets to ensure that nutrient requirements are met and to achieve maximum fish growth (Watanabe et al., 1988).

Mineral requirements for zebrafish have not been determined but are assumed to be similar to those of other cyprinid fish (Watts et al., 2012). Survival, body weight, lipid and protein content of zebrafish that were housed in zinc supplemented water and fed a zinc supplemented diet were found to be no different from control fish, however, supplementation did cause stimulation of stem cell differentiation in the gills (Zheng et al., 2010). Limiting the amount of calcium in the diet of zebrafish and maintaining water calcium levels below 1 ppm was found to have no effect on growth of zebrafish (Padgett-Vasquez et al., 2008).

Vitamins

Vitamins are classified as either water-soluble or fat-soluble. Water-soluble vitamins include those in the vitamin B complex, choline, inositol and vitamin C. Some warmwater fish are able to obtain water-soluble vitamins from microorganisms in the gastrointestinal tract (Limsuwan and Lovell, 1981; Shi-Yen and Lung, 1993), while coldwater fish have no such ability (Hepher, 1988). Fat-soluble vitamins include vitamins A, D, E and K. Quantitative vitamin requirements have been estimated for several species of fish (National Research Council, 2011). Vitamin deficiencies can lead to many sub-lethal effects including abnormal swimming, decreased liver lipids, deformities, edema, lethargy, lordosis and scoliosis. Similar to minerals, vitamin supplement mixes are often added to diets. However, some vitamins such as ascorbic acid quickly degrade or leach into the water when diets are fed to fish. To compensate for these actions supplemental vitamin mixes are often over-formulated in order to ensure that nutrient requirements are met.

While vitamin requirements for zebrafish have not been determined, current thinking is that vitamin requirements are similar to other cyprinid species (Watts et al., 2012). Vitamin E deficient diets have no effect on growth of adult zebrafish but embryos produced from those fish demonstrated an increase in abnormalities and mortality (Miller et al., 2012). In terms of growth rate and fecundity, diets that contained 1000 mg ascorbic acid kg^{-1} diet were found to be optimal for zebrafish compared to diets containing 250, 500 and 2000 mg ascorbic acid kg^{-1} (Mehrad et al., 2011).

Carbohydrates

In general, fish do not have a specific requirement for carbohydrates in their diets. It is assumed that glucose is efficiently synthesized from amino acids via gluconeogenesis (Walton and Cowey, 1982; Suarez and Mommsen, 1987; Cowey and Walton 1989). Most fish feeds include a relatively small amount of carbohydrates compared with feeds for poultry or mammals. Carbohydrate digestibility varies greatly depending on the composition, processing and level of inclusion (National Research Council, 2011). Carbohydrate inclusion into fish diets is often used to spare the use of lipids and proteins as an energy source, and hence save money. However, persistent hyperglycemia can be an issue when fish are fed highly digestible carbohydrates (National Research Council, 2011). Teleost (bony) fish, which include zebrafish, have been shown to be slow at clearing glucose from their bloodstream and are often considered glucose intolerant (Moon, 2001).

Most fish species have α-amylase present in their digestive tract, which can break the α-glycosidic bonds of starch making it digestible (Jobling, 1995; National Research Council, 2011). Activity level of the α-amylase often corresponds to the trophic status of the species, with low activity level pertaining to carnivorous fish, medium levels for omnivorous fish and high levels for herbivorous fish (Stone et al., 2003). Starch digestibility is also affected by molecular complexity, starch origin, degree of gelatinization and dietary inclusion level (Krogdahl et al., 2005; National Research Council, 2011). Larval zebrafish diets with starch inclusion of 25% displayed the best growth compared with 0, 15 and 35% inclusion, however no difference was found between 15, 25 and 35% inclusion (Robison et al., 2008).

Non-starch polysaccharides (NSP) are a poor source for energy in fish. A reduction of growth was reported in several species when NSP inclusion exceeded 10% (Hilton et al., 1983, Shiau et al., 1988; Dioundick and Stom 1990). Galactose is also indigestible to fish as they lack the α-galactosidase enzyme (Glencross et al., 2003). The same is true for cellulose because fish lack the enzyme cellulase. (National Research Council, 2011). Therefore, cellulose is often used as a bulking agent when formulating feeds.

It is recommended that levels of digestible starch inclusion into diets not exceed 15 to 25% for salmonids and 50% for omnivorous species while dietary fiber levels should be kept as low as possible and should not exceed 10% (National Research Council, 2011).

Protein and Amino Acids

Proteins and amino acids (AA) play important roles in structure and metabolism of all animals (National Research Council, 2011). In fish, protein accounts for approximately 65 to 75% of their total dry weight (Wilson, 2002). There are thousands of different proteins that play integral roles in fish and each has its own specific structure and amino acid sequence. Proteins such as collagen and elastin play a role in connective tissue, while myosin has an important critical role in muscle contraction. Other proteins are critical for catalyzing chemical reactions, cell signaling, immune response and can act as transporters.

While fish do not have a true protein requirement, a well-balanced mixture of AA is required (Wilson, 2002). There are 20 primary AA that are used in protein

biosynthesis (National Research Council, 2011). Although fish can synthesize some of these AA, often referred to as non-essential amino acids (NEAA), other AA need to be supplied in the diet and are referred to as essential amino acids (EAA). All fish species that have been studied require the same 10 EAA including: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (National Research Council, 2011). A deficiency or elimination of one of these EAA can result in a reduction in animal growth performance (National Research Council, 2011) as protein is taken from less crucial tissues in order to maintain those that are more important (Wilson, 2002). Each individual EAA requirement varies between species so in order to formulate a diet that will achieve optimal growth and/or performance, EAA requirements of each species must be determined. While EAA and protein requirements for zebrafish have not yet been determined, most cyprinids require between 30 to 53% protein in their diet (Ulloa et al., 2011).

During digestion, proteins are broken down into polypeptides, tripeptides, dipeptides and free amino acids via hydrolysis. These products are then absorbed by the mucosal cells where it is presumed that intracellular digestion occurs as only free amino acids are released into the portal vein (Murai et al., 1987). These amino acids then enter the free amino acid pool where they can be used for protein synthesis or catabolism. McLean et al. (1999) presume that some intact proteins can be absorbed through the wall of the gastrointestinal tract, however it is assumed to be a small quantity and most likely serves a role in the immune system (McLean et al., 1999).

Energy production via amino acid oxidation is common in fish, whether the amino acids are in excess or not. A certain fraction of amino acids that are absorbed are

inevitably catabolized to produce energy as well as byproducts such as ammonia, carbon dioxide and bicarbonate. Ammonia is very toxic and in mammals is often converted to urea to prevent toxicity. Fish are in a unique situation because they can efficiently excrete ammonia through their gills and not waste energy converting it to urea. Most fish excrete more than 80% of their nitrogenous wastes in ammonia form (Kaushik and Cowey, 1991). Although this process is advantageous from an energy standpoint, feeding fish an excessive amount of protein can result in an increase in ammonia production and cause water quality degradation, an important factor in raising healthy fish.

Lysine

Lysine is found abundantly in body protein of many animals including fish (National Research Council, 2011). Myosin and tryptomyosin were found to contain more than 14% lysine (Pellet and Young, 1984). Derivatives of lysine such as hydroxylysine and allysine are essential to forming crosslinks that stabilize collagen (Sassi, 2001), which is a major component of connective tissue. Lysine is often the first limiting amino acid in feeds for warmwater fish (Robinson et al., 1980). Ingredients such as fishmeal and blood meal contain relatively high concentrations of lysine while plant proteins are often deficient in lysine. Excessive heating and nonenzymatic glycolyzation of these feedstuffs during feed manufacturing causes the formation of Maillard reaction products, resulting in a reduction of available lysine in feeds (Carpenter, 1960; Moughan and Rutherford, 1996). A deficiency of lysine in the diet can cause a reduction in growth

and feed efficiency in fish (National Research Council, 2011) as well as dorsal and caudal fin erosion (Ketola, 1983; Guillaume et al., 1999).

Of the 10 essential amino acids (EAA) needed by fish, the lysine requirement is the highest (National Research Council, 2011), ranging between 4 and 5% of protein for most fish (Wilson, 2002). It is suspected that zebrafish are no exception, as lysine was found in the highest concentration of all 10 EAA in the whole body amino acid profile of zebrafish (Gomez-Requeni et al., 2010; Kaushik et al., 2011).

Arginine

Arginine is considered a semi-essential or conditionally essential amino acid in mammals (Baker, 2007). Arginine is an intermediate of the urea cycle, however, urea cycle activity in fish is poor and thus arginine is considered an EAA for all fish species (National Research Council, 2011). The urea cycle is not as prevalent in fish because they can easily discard excessive nitrogen as ammonia through the gills as previously mentioned. Arginine is a precursor to nitric oxide and creatine and acts as a stimulant of insulin and growth hormone (Wan et al., 2006).

Arginine requirements for fish commonly range between 4 and 5% of protein in the diet (Wilson, 2002) with Chinook salmon (*Oncorhynchus tshawytscha*) requiring as much as 6.0% (Klein and Halver, 1970) and wels catfish (*Silurus glanis*) needing only 3.4% (Toth, 1986). The ideal protein profile for zebrafish determined by Kaushik et al. (2011) showed that the arginine requirement for zebrafish is estimated to be 78% of that for lysine (Kaushik et al., 2011). This value, along with the zebrafish quantitative lysine requirement can be used to calculate a rough estimate for arginine requirement of

zebrafish. A deficiency in arginine can cause a reduction in growth in fish and may have a negative effect on fish health (Gatlin, 2002).

Lysine and Arginine Interactions

Because lysine and arginine share the same transporter, lysine-arginine antagonism has been found in several animal species including chicks, rats, guinea pigs and dogs (National Research Council, 2011), however there is some debate whether this occurs in fish species (Kaushik and Fauconneau, 1984; Ren et al., 2013). It has been suggested that antagonism exists between lysine and arginine in rainbow trout (*Oncorhynchus mykiss*) (Kaushik and Fauconneau, 1984) and Atlantic salmon (*Salmo salar*) (Berge et al., 1997; Berge et al., 1998). Studies with other species such as channel catfish (*Ictalurus punctatus*) (Robinson et al., 1981), European sea bass (*Dicentrarchus labrax*) (Tibaldi et al., 1994) and Japanese flounder (*Paralichthys olivaceus*) (Alam et al., 2002) exhibited no such interaction.

QUANTITATIVE AMINO ACID STUDIES IN FISH

Zebrafish nutrition research should be similar to research for the finfish and shrimp aquaculture industry (Watts et al., 2012). Previous research conducted on zebrafish nutrition should more appropriately be termed “dietetics” because these studies evaluated common diets and provided important information regarding basic feeding strategies, but were not adequate for quantitative nutrient requirement assessment (Watts et al., 2012).

Whole-body amino acid analysis analyzes the amino acid profile of body protein and can be used as an initial estimate of the amino acid requirements for zebrafish (Mambrini and Kaushik, 1995; Kaushik, 1998; Kaushik and Seliez, 2010). A good relationship has been found between EAA in the whole-body and their actual requirement in channel catfish (Wilson and Poe, 1987) and common carp (Nose, 1979). However, amino acids are used differently in the body. The AA lysine and leucine are highly retained in tissue while methionine, threonine, histidine and arginine have greater metabolic roles (National Research Council, 2011). Whole-body amino acid analysis has been conducted on zebrafish (Gomez-Requeni et al., 2010; Kaushik et al., 2011) and was shown to have a similar profile to other cyprinids (Kaushik et al., 2011). Requirement values of AA estimated from whole-body analysis are often lower than those determined by growth studies (Wilson, 2002). Establishing quantitative estimates of EAA requirements for fish is most often done by dose-response studies (Mambrini and Kaushik, 1995; National Research Council, 2011). While there are many ways to express EAA requirements such as percent of protein, grams MJ^{-1} digestible energy and percent of diet, each method of expression has its own limitations. The NRC prefers expressing EAA requirements on a percent dry matter basis (National Research Council, 2011).

Diets

Most studies that evaluate EAA requirements use a basal diet deficient in a single target EAA but meets or exceeds requirements of all the other nutrients for that species (National Research Council, 2011). Test diets are made by adding graded levels of the target amino acid, often in crystalline amino acid (CAA) form, to the basal diet (National

Research Council, 2011). The number of test diets (treatments) for a quantitative nutrient requirement study should be at least five (National Research Council, 2011). The basal diet should provide a baseline level for evaluating the response variables while the other test diets should have concentrations ranging from deficient to excessive for the expected requirement (National Research Council, 2011). The number of replicates used in nutrient requirement studies can vary but is often done in triplicate (Santiago and Lovell, 1988; Ahmed and Khan, 2004; Wang et al., 2005; Lin et al., 2013; Ren et al., 2013). Test diets are often made isonitrogenous by decreasing the levels of NEAA such as aspartic and/or glutamic acid (Wilson et al., 1977; Lin et al., 2013). In order to provide maximum control over nutrient composition of the diets, semipurified diets consisting of ingredients with defined chemical compositions should be used (National Research Council, 2011). Protein sources for these basal diets often come from casein and/or gelatin (Wilson, 2002).

High levels of CAAs may lower the pH of feed, but adjusting the pH with sodium hydroxide was found to increase utilization of these diets (Nose et al., 1974; Wilson et al., 1977; Murai et al., 1981). Crystalline amino acids have also been shown to be more rapidly absorbed than intact protein-bound amino acids (Yamada et al., 1981; Murai et al., 1981; Kaushik et al., 1983; Cowey and Walton, 1988; Tantikitti and March, 1995; Zarate and Lovell, 1997; Zarate et al., 1999). Coating, encapsulation or polymerization of CAAs has been demonstrated to reduce the absorption rate and improve utilization of CAAs in fish (Murai et al., 1981; Teshima et al., 1990; Cho et al., 1992; Dabrowski et al., 2003; Segovia-Quintero and Reigh, 2004; Zhou, 2007; Dabrowski et al., 2010).

In most EAA requirement studies, the EAA that are supplied in the test diets were nearly 100% digestible (National Research Council, 2011). There has been no research on feedstuff digestibility for zebrafish conducted to date. While this information is lacking, it has been implied that digestible protein values of practical ingredients are similar between species (Bureau et al., 2002).

In general, dietary nutritional experiments conducted on zebrafish should achieve at least 90% survival to be considered viable (Watts et al., 2012). Some species may elicit a reduction in growth and intake when fed test diets, so it is important to include a reference diet in these experiments which will support normal weight gain in order to estimate the growth potential of the species (National Research Council, 2011). While most reference diets are composed of practical ingredients, live food diets may also be used (National Research Council, 2011). Data derived from reference diets can be used to compare performance to experimental diets, but should not be included when quantifying a nutrient requirement (National Research Council, 2011).

Presentation of the diet can be important to some fish as some species only feed on the bottom, top or middle of the water column. Zebrafish are not as particular as some species because they have been observed feeding in every part of the water column (Spence et al., 2007) and will readily consume diets that are manufactured for floating or sinking (Watts et al., 2012). It is ideal for each ingredient in the experimental diet to be from the same homogenous lot with each ingredient ground to a standard size to ensure homogeneity after mixing (National Research Council, 2011). Nutrient analysis of the experimental diets must be done before the start of the feeding trial. It is important to

report results from the feeding trial according to the analyzed nutrient levels and not calculated levels derived during formulation (National Research Council, 2011).

Response Variables

It is estimated that 25 to 55% of amino acids that are consumed are deposited into body protein (National Research Council, 2011), and protein deposition is considered a major determinant of amino acid utilization and requirement of fish (Cowey and Walton, 1989). Because there is a strong association between protein deposition and weight gain in fish (Shearer, 1994; Dumas et al., 2007), the most common response variable for quantitative nutrient requirement studies is weight gain (Baker, 1986; Mambrini et al., 1995; Wilson, 2002). However, general growth is typical (National Research Council, 2011; Watts et al., 2012) which can include length measurements as well (Watts et al., 2012). For smaller more rapidly growing fish, such as zebrafish, the duration of feeding trials should be long enough to observe a change in weight of up to 1,000% of the initial weight (National Research Council, 2011).

Feed conversion can also be used as a response variable, which can include feed conversion ratio or feed efficiency (National Research Council, 2011). This method of expression requires accurate estimates of feed consumption via calculating the amount of feed given and the amount of uneaten food. Zebrafish size and the quantity of food given makes it difficult to calculate consumption (Watts et al., 2012) and therefore is not an optimal response variable.

Nutrition has an effect on reproduction and egg quality in several species including zebrafish (Laale, 1977; Meinelt et al., 1999; Markovich, 2007), carp

(Watanabe, 1985), yellow perch (*Perca flavescens*) (Kwasek et al., 2014), and rainbow trout (Takeuchi et al., 1980; Watanabe, 1985). Zebrafish are prized for their ability to produce eggs (Lawrence 2011), fecundity is an important response variable that should be examined (Kaushik et al., 2011; Watts et al., 2012).

Blood and muscle amino acid levels can also be used as a response variable. Serum or tissue content of the target EAA should be low if the intake is below the required levels (Wilson, 2002) and should be high if intake is in excess of the requirement (Wilson, 2002; National Research Council, 2011). While serum EAA levels are not always reliable predictors of their requirement (Kaushik and Luquet, 1979; Hughes et al., 1983; Walton et al., 1986; Kim et al., 1992), serum EAA levels have successfully confirmed the lysine requirement in channel catfish fed a diet at 24% crude protein (Wilson et al., 1977). It should be noted that this method was not successful when channel catfish were fed a diet at 30% crude protein diet (Robinson et al., 1980). Serum EAA levels have not yet been measured for zebrafish.

Response data is most often evaluated by using a broken-line regression model (Wilson, 2002; National Research Council, 2011). Mean separation tests such as ANOVA are not the appropriate statistical analysis tool for dose-response quantitative studies because they do not estimate the nutrient requirement (National Research Council, 2011). The broken line model is a linear model that assumes the response increases as the amount of the limiting nutrient increases, up until the point where the requirement is met. After this point, increasing the amount of the limiting nutrient will result in no change in the response or perhaps show a decrease (Wilson, 2002; National Research Council, 2011). The break point of the two resulting linear lines is the estimate

of the nutrient requirement. This model works well for quantitative amino acid studies, as weight gain is apparently linearly related , but this model often provides lower requirement estimates than other models (National Research Council, 2011). Other non-linear models, including exponential, quadratic and sigmoidal models, have been used when data does not properly fit the broken-line model (Wilson, 2002; Ahmed and Khan, 2004; Wang et al., 2005; National Research Council, 2011). Regardless of which model is used, it is important that all data points from each replicate of each treatment are used, not just the mean data points for each treatment (National Research Council, 2011).

Dietary Lysine and Arginine Requirements of Juvenile and Adult Zebrafish (*Danio rerio*)

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INTRODUCTION

Use of zebrafish as a model organism in the fields of toxicology, developmental biology, pharmacology, neurology, biochemistry and cell biology has increased drastically in the past 20 years (Kinth et al., 2013). Many biological characteristics such as high fecundity, short generation time, predictable spawning time and transparent embryos as well as their low-cost and their ability to withstand a wide range of culture conditions have attributed to their increase in popularity.

While optimal culture conditions such as water temperature and water chemistry values have been established for zebrafish, development of a standardized diet has lagged behind. Failure to control for dietary variables can influence experimental outcomes in animal models (Lawrence, 2007; Siccardi et al., 2009; Kent and Varga, 2012; Penglase et al., 2012; Watts et al., 2012). In order for a standardized diet to be adequate, the feed must meet or exceed the zebrafish's macronutrient requirements (Ahmed and Khan, 2004; Ulloa et al., 2011; Watts et al., 2012; Smith et al., 2013). To date, there has been no published data on any of the macronutrient requirements of zebrafish.

While fish do not have an overall protein requirement, a well-balanced mixture of amino acids (AA) is required (Wilson, 2002). Of the 20 primary AA that are used in protein biosynthesis, 10 are considered essential amino acids (EAA). Of these 10 EAA, lysine and arginine are required in the highest concentrations for all fish (National Research Council, 2011). Lysine is found abundantly in the body protein of many animals including fish (National Research Council, 2011). Derivatives of lysine such as hydroxylysine and allysine are essential to forming crosslinks that stabilize collagen (Sassi, 2001), which is a major component of connective tissue. Arginine is a precursor

to nitric oxide and creatine which act as a stimulant of insulin and growth hormone (Wan et al., 2006).

The objective of these experiments was to determine dietary lysine and arginine requirements for juvenile and adult zebrafish.

MATERIALS AND METHODS

This study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota (IACUC Protocol #1309-30934A). Wild-type (Segrest Farms, Gibsonton, FL) zebrafish were used exclusively for these experiments. Zebrafish were maintained in a commercially available recirculating aquaculture system (Aquaneering, San Diego, CA). Photoperiod was set at 14 h of light and 10 h of a dark cycle. Water temperature and water chemistry values were maintained within acceptable ranges shown in Tables 1 and 2.

Experiment 1 - Lysine

Diet Preparation and Experimental Diets

Seven purified experimental diets were utilized in this experiment. Experimental diets (Table 3) were formulated to meet or exceed the requirements of common carp (National Research Council, 2011) except for lysine. L-lysine was added in varying amounts (0 to 16 g kg^{-1}) in order to obtain dietary lysine values above and below what was thought to be the requirement. Levels of glutamic acid were adjusted to ensure diets were isonitrogenous and isocaloric.

Diet preparation was similar to Garling and Wilson (1976). Crystalline amino acids (CAA) were homogenized with 4.0 g of carboxymethyl cellulose (CMC). Fifty mL of 50^{o}C distilled water were added to make the mixture gelatinous. In a separate container, casein, gelatin, dextrin, alpha cellulose and the remaining CMC was mixed together by hand until homogenous. The CAA/CMC paste was then added to the dry mixture and mixed thoroughly, followed by a slow addition of 200 mL of 50^{o}C distilled water. The mixture was allowed to cool for a few minutes before vitamin and mineral mixes were added and mixed thoroughly. Menhaden oil was then slowly added and mixed thoroughly. Adjusting pH of the diets was similar to that of Wilson et al. (1977) where 6N NaOH was added until pH reached approximately 7. To accomplish this task, a 5 g sample was mixed with 50 mL of distilled water. The pH of the resulting supernatant was then measured. Diets were put through a food chopper (Universal Gourmet Food Chopper Model #2, Universal Trading Company, USA) to form pellets, placed into a 60^{o} C dryer (General Signal Company, Blue M Electric, USA) for one hr, stirred and dried again for another two hr. Resulting diets were put into Ziploc bags and placed in a freezer. Before feeding, diets were ground to appropriate size utilizing a coffee grinder (Mr. Coffee, Model BVMC-BMH23, Sunbeam Products, Cleveland, Ohio).

Juvenile Study

Juvenile zebrafish, 43 days post-fertilization (dpf), were randomly assigned to one of seven dietary treatments. Each treatment consisted of three tanks (2.8 L) with 10 fish in each tank. Previous to the start of the experiment, fish were fed 0.03 g of decapsulated

brine shrimp cysts (Brine Shrimp Direct, Ogden, UT) twice daily and 0.12 g of commercial pellet food (Scientific Hatcheries Diet for Zebrafish, Aquaneering, San Diego, CA) three times per week. Fish were transitioned onto their respective experimental diets over a five-day period by feeding 0.08 g of their experimental diet in the morning and 0.03 g of decapsulated brine shrimp cysts (Brine Shrimp Direct, Ogden, UT) in the evening. After this period, they were exclusively fed 0.08 g of their experimental diet twice daily until 90 dpf. Mortality was recorded weekly. At 90 dpf, zebrafish were anesthetized using Tricaine (Sigma, St. Louis, MO) and placed on a ruler for measuring fork length to the nearest millimeter. Fish were then patted dry with a paper towel and weighed to the nearest 0.001 g (Ohaus TS200S, Parsippany, NJ).

Adult Study

Adult zebrafish between 90 and 170 dpf that had never been set up to spawn, were randomly assigned to one of seven dietary treatments. Each treatment consisted of three tanks (2.8 L) with 10 fish (five males and five females) in each tank. Previous to the start of the experiment, fish were fed decapsulated brine shrimp cysts (Brine Shrimp Direct, Ogden, UT) beyond satiation twice daily and a commercial pellet food (Scientific Hatcheries Diet for Zebrafish, Aquaneering, California, USA) beyond satiation three times per week. Zebrafish were transitioned onto their respective experimental diets over a six-day period by feeding 0.12 g of their experimental diet in the morning and 0.03 g of decapsulated brine shrimp cysts (Brine Shrimp Direct, Ogden, UT) in the evening. After this 6-d period, they were exclusively fed 0.12 g of their experimental diet twice daily. Mortality was recorded weekly.

Starting on week 4 of the study, each tank of fish was placed into a breeding container overnight for spawning once per week. All treatments were set up for breeding on the same day at the same time and all embryos were harvested in the afternoon. Viable embryos were collected, counted, and recorded for each spawning event. Embryos were considered viable if they were shown to be developing 4h after the lights turned on in the fishroom.

Plasma was collected on week 12 of the study. Blood collection was similar to that previously described by Liqing et al. (2013). Fish were fasted overnight and anesthetized with 0.4% Tricaine just before collection began. Glass capillary tubes (World Precision Instruments Inc. 1B100F-4, Sarasota, FL) were pulled using a needle puller (Sutter Instrument Co. Model P-87 Needle Puller, Novato, CA) to form micro-capillary needles. Needles were then coated with heparin. Blood was collected by inserting the heparinized needle into the caudal region, behind the anus, near the caudal vein and dorsal aorta. Enough blood was collected and pooled for one sample per treatment. Pooled blood was placed into a 10 μL heparinized capillary tube, capped, and placed into a 15 mL centrifuge tube for centrifugation. Samples were spun (Hettich Mikro 22R, Tuttlingen, Germany) at 4^{o}C at 2230 RFC for 5 minutes and plasma was then mixed with heparin and frozen to -80^{o}C.

Experiment 2 – Arginine

Experimental Diets and Diet Preparation

Seven experimental diets (Table 4) were formulated to meet or exceed the requirements of common carp (National Research Council, 2011), except for arginine.

L-arginine was added in varying amounts in order to obtain dietary arginine values above and below what was thought to be the requirement. Levels of glutamic acid were adjusted to ensure diets were isonitrogenous and isocaloric. Diet preparation was similar to what was described in Experiment 1.

Juvenile Study

Juvenile zebrafish, 42 days post-fertilization (dpf), were randomly assigned to one of seven dietary treatments. Protocols for this study are the same as described in Experiment 1.

Adult Study

Adult zebrafish between 112 and 175 dpf that had never been set up to spawn, were randomly assigned to one of seven dietary treatments. Protocols for this study are the same as described in Experiment 1 with the exception of plasma collection.

Plasma was collected on week 10 of the study. Blood collection was similar to that previously described by Liqing et al. (2013). Glass capillary tubes (World Precision Instruments Inc. 1B100F-4, Sarasota, FL) were pulled using a needle puller (Sutter Instrument Co. Model P-87 Needle Puller, Novato, CA) to form micro-capillary needles. Needles were then coated with heparin. Zebrafish were fasted overnight, euthanized and decapitated. Blood was collected from the site of decapitation using a heparinized needle. Enough blood was collected and pooled for three samples per treatment. Pooled blood was placed into a 10 μL heparinized capillary tube, capped, and placed into a 15 mL centrifuge tube for centrifugation. Samples were spun (Hettich Mikro 22R,

Tuttlingen, Germany) at 4°C at 2230 RFC for five minutes and plasma was then mixed with heparin and frozen to -80°C.

Chemical Analysis

Proximate composition of experimental diets was estimated using standard Association of Official Analytical Chemists (AOAC) procedures for moisture (934.01 vacuum oven), crude protein (984.13 Kjeldahl), ash (942.05), crude fat (954.02 acid hydrolysis) and crude fiber (978.10) (Horwitz, 2006). Gross energy was determined by bomb calorimetry (PARR 1281 Bomb Calorimeter, Parr Instrument Company, Moline, IL). Amino acid analysis was determined with standard AOAC method 982.30E (a,b,c) (Horwitz, 2006).

Serum AA were determined using liquid chromatography-mass spectrometry (LC-MS) analysis. Serum samples were prepared by mixing one volume of serum, one volume of 100 μM p-chlorol-L-phenylalanine (internal standard), and 18 volumes of 66% aqueous acetonitrile, followed by centrifugation at 18,000 × g for 10 min to obtain the supernatant. Five μL of deproteinized sample were mixed with 40 μL of Na_2CO_3 (10 mM, pH 11) and 100 μL of dansyl chloride (3 mg/mL in acetone). The mixture was incubated in a water bath at 60° C for 10 min followed by centrifugation at 18,000 × *g* for 10 min at 4° C. The supernatant was transferred to a HPLC vial and a 5 μl of aliquot was injected into an Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA) and separated in a BEH C18 column using a mobile phase gradient ranging from water to 95% aqueous acetonitrile containing 0.1% formic acid over a 10 min run. Liquid chromatography effluent was introduced into a Xevo-G2-S

quadrupole time-of-flight mass spectrometer (QTOFMS, Waters) for accurate mass measurement and ion counting. Capillary voltage and cone voltage for electrospray ionization was maintained at 3 kV and 30 V for positive-mode detection. Source and desolvation temperatures were set at 120 and 350°C, respectively. Nitrogen was used as a cone gas (50 L/h) and desolvation gas (600 L/h), and argon as collision gas. To attain accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution (range m/z 50-1,000) and monitored by the intermittent injection of the lock mass leucine enkephalin ([M+H]+ = m/z 556.2771) in real time. Mass chromatograms and mass spectral data were acquired and processed by MassLynx^{TM} software (Waters, Milford, MA) in centroided format. Individual AA concentrations were determined by calculating the ratio between the peak area of AA and the peak area of internal standard and fitting with a standard curve using QuanLynx^{TM} software (Waters, Milford, MA).

Statistical Analysis

Final weight, final length, embryo production and plasma amino acids were subjected to one-way ANOVA followed by Tukey's Range test to determine significant differences among treatment groups. Statistical significance was set at $P < 0.05$. Broken-line linear regression and quadratic regression models (R Statistical Computing) were used to estimate zebrafish lysine and arginine requirements based on final weight, final length and embryo production.

RESULTS

Experiment 1

Survival rates for all treatments were generally high (Tables 5 and 6), with tank survival ranging from 83 to 100%. The highest survival was observed in zebrafish that were fed 1.42 and 2.26% dietary lysine while the lowest survival was found in zebrafish fed 1.73% dietary lysine.

Juvenile zebrafish fed diets containing 2.01% dietary lysine were determined to have the highest mean final weight (Table 5), however there was no difference ($P > 0.05$) among treatments. The lowest mean final weight was found in zebrafish fed 1.73% dietary lysine. Analysis of the final weight data using broken-line regression revealed a linear increase in performance as dietary lysine increased up until 2.21% dietary lysine, after which a decrease in performance was observed (Figure 1). Similarly, the quadratic regression model shows peak mean final weight to be achieved at 2.20% dietary lysine (Figure 2).

Mean final lengths for juvenile zebrafish were different ($P < 0.05$) between treatments (Table 5). Zebrafish fed diets containing 2.16% dietary lysine exhibited the highest mean final length while zebrafish fed diets containing 1.73% dietary lysine had the lowest mean final length. A broken-line regression model was unsuccessful at estimating a break-point with this data while the quadratic regression model suggests optimal performance to be at 2.47% dietary lysine (Figure 3).

Mean embryo production was different ($P < 0.05$) between treatments (Table 6) with zebrafish fed diets containing 2.16% dietary lysine generating the most embryos. Mean embryo production ranged between 68.3 and 436.3 embryos. Broken-line

regression estimates the break-point to be 2.16% dietary lysine (Figure 4). Quadratic regression analysis differed by predicting the highest embryo production to peak at 1.79% dietary lysine (Figure 5).

Plasma lysine concentrations were highest in zebrafish fed diets containing 2.57% dietary lysine, followed closely by zebrafish fed 2.26% dietary lysine (Table 7). Plasma lysine concentrations were relatively similar among treatments ranging from 124 to 146 uM, with the exception of 1.73% dietary lysine where the concentration dropped to 83.3 uM.

Experiment 2

Similar to Experiment 1, survival rates for all treatments were generally high (Tables 8 and 9), with tank survival ranging from 93 to 100%. The highest survival was observed in zebrafish that were fed 0.80, 1.56 and 1.88% dietary arginine while the lowest was found in zebrafish fed 1.08, 2.37 and 2.70% dietary arginine.

No differences ($P > 0.05$) were observed among treatments for both mean final weight and final length (Table 8). Neither mean final weight nor final length data were able to fit into broken-line regression models. A general decrease in performance was observed as the amount of dietary arginine increased, with the exception of 2.70% dietary arginine. These results using quadratic regression models for both data sets gave an uncharacteristic trend-line for dose-response nutritional studies (Figures 6 and 7).

Mean embryo production did not differ ($P > 0.05$) among treatments (Table 9). Broken-line regression analysis estimated the break-point to be 1.95% dietary arginine

(Figure 8) while quadratic regression analysis estimated peak performance was achieved at 1.07% dietary arginine (Figure 9).

Mean plasma arginine concentrations did not differ ($P > 0.05$) among treatments (Table 9). Broken-line regression projected a break-point at 1.43% dietary arginine (Figure 10) while quadratic regression revealed peak performance to be at 1.73% dietary lysine (Figure 11).

DISCUSSION

Experiment 1

A survival rate of at least 80% is thought to be optimal for nutritional experiments using zebrafish (Watts et al., 2012). Tank survival in the current study was typically between 90 and 100% (Tables 5 and 6) with only five of 42 tanks having 80% survival, indicating survival was adequate for nutrient requirement research.

Results from the current study indicate that optimum growth and embryo production in zebrafish occurs when fed a diet consisting of 2.2% dietary lysine. This observation is based upon broken-line and quadratic regression models for weight (2.21% and 2.20%) as well as the broken line model for embryo production (2.16%). Quadratic regression analysis based on length data suggests a higher dietary lysine (2.47%) requirement, however weight gain is more closely correlated with protein deposition (Shearer, 1994; Dumas et al., 2007) and is the most common response variable for quantitative nutrient requirement studies (Baker, 1986; Mambrini et al., 1995; Wilson, 2002). Quadratic regression analysis of embryo production data showed a lower dietary lysine requirement (1.79%) than broken-line regression analysis (2.16%), however non-

linear models are used only when data does not properly fit the broken-line model (Wilson, 2002; Ahmed and Khan, 2004; Wang et al., 2005; National Research Council, 2011). Based on results from the current experiment, the optimum dietary lysine requirement for juvenile and adult zebrafish is recommended to be 2.2% of dry diet which corresponds to 5.6% of dietary protein.

Similar dietary lysine requirements were found for other cyprinid or omnivorous species such as common carp (2.2% of dry diet and 5.70% of protein (Nose, 1979)), juvenile grass carp (2.24% of dry diet and 5.89% of protein (Wang et al., 2005)), fingerling Indian major carp (2.30% of dry diet and 5.75% of protein (Ahmed and Khan, 2004)) and juvenile cobia (2.3% of dry diet and 5.30% of protein (Zhou et al., 2007)). Dietary lysine requirements of zebrafish differed from species such as juvenile yellow catfish (3.3% of diet and 8.3% of protein (Cao et al., 2012)), tilapia (1.6% of diet and 4.1% of protein (Jackson and Capper, 1982)), nile tilapia (1.4% of diet and 5.1% of protein (Santiago and Lovell, 1988)) and black sea bream (3.3% of diet and 8.6% of protein (Zhou et al., 2010)). These species are known to be either herbivores, carnivores, or inhabit cold-water environments. Zebrafish are a warm-water omnivorous species, which could explain differences in requirements by various species of fish.

Using quantitative lysine data obtained from the present study in combination with the ideal protein profile of zebrafish established by Kaushik et al. (2011), quantitative requirements for the other nine EAA was estimated (Table 10). However, further quantitative nutritional research is recommended because the ideal protein profile should only be used as a starting point for such research (Kaushik et al., 2011).

Growth from zebrafish fed experimental diets was lower ($P < 0.05$) than those fed the reference diet (Figures 12 and 13). While optimal growth is not expected with the experimental diets, the difference in performance between experimental diets and the reference diet is disconcerting. Growth rates from experimental diets should be at least 75% of growth rates for fish living in natural environments under comparable conditions (Watts et al., 2012). Growth in the present experiment is similar to that of zebrafish found in their natural environment (Spence et al., 2007), however growth conditions cannot be considered comparable.

Some fish species do not accept casein-gelatin diets (National Research Council, 2011) due to a decrease in palatability. However, zebrafish in the present experiment were observed consuming experimental diets immediately after it was dropped in the tank, suggesting that it is palatable. Extra feed was also found at the bottom of the tank so it is possible that the zebrafish are consuming enough to satiate themselves but are not absorbing enough nutrients to meet their needs for growth and embryo production. Unfortunately, digestibility of feed ingredients for zebrafish is unknown. Apparent digestibility of casein, gelatin, crystalline amino acids and menhaden oil are generally high for most species of fish (Halver and Hardy, 2002; National Research Council, 2011). Digestion of carbohydrates can be variable between species, depending on α–amylase activity. Zebrafish are considered omnivorous and as a result are thought to have moderate α-amylase activity (Stone et al., 2003). However, this is a generalization and it is possible that dextrin may be relatively indigestible.

While carbohydrates are not required in fish diets, they can be used as a source of energy. If digestible energy is lacking in the experimental diets due to indigestible

carbohydrates, increasing the protein or lipid content of the diet could increase the digestible energy content. It is recommended that future experimental diets contain higher lipid content in order to improve palatability and growth.

It should be noted that the small size of zebrafish made it difficult to collect blood. A non-lethal procedure was used to draw blood in this experiment, thus extra care was taken to avoid extracting too much blood from an individual fish. Zebrafish blood has also been known to clot in under 10 seconds (Jagadeeswaran et al., 1997). These technical issues resulted in only one pooled-sample to be obtained for each dietary treatment. While this data is incomplete and difficult to interpret, this is the first time plasma amino acid concentrations have been measured in zebrafish. It is worth noting that plasma lysine levels of the dietary treatments were higher than that of the reference diet (Figure 14). Whether high plasma lysine is due to insufficient lysine concentration in the diet or possible antagonism by other AA is unknown. Future blood collection methods should be modified in order to obtain multiple samples per dietary treatment.

Experiment 2

Similar to Experiment 1, survival rates in the present experiment (Tables 8 and 9) were adequate for studying nutrient requirements.

Broken-line and quadratic regression models based on final weight and final length data were unable to determine the dietary arginine requirement. Broken-line regression models did not fit the data and quadratic regression models depicted an uncharacteristic result (Figures 6 and 7). Quadratic models were expected to elicit an increase in the response variable as the dosage approaches the requirement, followed by a

decrease in the response variable as that requirement is exceeded. The models in the present experiment show the inverse of what was expected. With the exception of 2.70% arginine, growth data sets indicate a general decrease in performance as dietary arginine concentration increases. Coincidentally, it was found that the experimental diet containing 2.70% arginine had the highest crude protein content (31.8%). While attempts were made to formulate isonitrogenous experimental diets, analyzed crude protein content varied from 27.7 to 31.8%. Lack of isonitrogenous diets may have contributed to the uncharacteristic data sets. It is possible that the dietary arginine requirement for juvenile zebrafish is below 0.8% of dry diet, as a general decrease in performance is observed from 0.8 to 2.37%. Further nutritional research with experimental diets containing arginine concentrations below 0.8% is recommended.

Embryo production data, using broken-line regression, indicate the dietary arginine requirement to be 1.95% of dry diet (Figure 8), which corresponds to 6.5% of dietary protein. This is slightly higher than what was estimated using the ideal protein profile (1.7%, Table 10) and what is required for common carp (1.7% of dry diet or 4.3% of protein (Nose, 1979)). Requirements for Nile tilapia (1.18% of dry diet and 4.20% of protein (Santiago and Lovell, 1988)), rainbow trout (1.4% of dry diet and 4.2% of protein (Cho et al., 1992)) and channel catfish (1.03% of dry diet and 4.29% of protein (Robinson et al., 1981)) were found to be much lower than the estimate for zebrafish. Arginine requirements for juvenile blunt snout bream (2.46% of dry diet and 7.23% of protein (Ren et al., 2013)) and coho salmon (2.2 to 2.5% of dry diet and 4.9 to 5.5% of protein (Luzzana et al., 1998)) were found to be higher.

Blood collection methods were modified for Experiment 2 in order to successfully obtain three pooled blood samples from each dietary treatment. Broken-line and quadratic regression models based on plasma arginine concentrations reveal the requirement to be 1.43 and 1.73% of dry diet, respectively. Plasma EAA concentrations have been used to confirm requirement growth data in the past (Wilson et al., 1977), however confirmation did not occur in the present study. Similar to other research (Robinson et al., 1981; Cho et al., 1992) plasma arginine concentrations remained relatively constant for each of the experimental diets which may be due to arginine being synthesized from other amino acids. Wan et al. (2006) stated arginine could be synthesized from citrulline, however, plasma citrulline concentrations do not indicate this occurrence (Figure 15). Proline has also been found to be synthesized to arginine in adult humans (Tomlinson et al., 2010) and neonatal piglets (Brunton et al., 1999). Arginine synthesis from proline may also be occurring in zebrafish because plasma proline concentrations decrease with a decrease in dietary arginine (Figure 16), while plasma arginine concentrations remain relatively stable.

Plasma lysine concentrations did not decrease due to an increase in dietary arginine (Figure 17), revealing that dietary arginine levels between 0.8 and 2.87% do not affect lysine absorption in enterocytes. Further research utilizing diets with varying lysine and arginine concentrations should be conducted to determine if antagonism exists.

Similar to Experiment 1, the present experiment showed lower ($P < 0.05$) growth in zebrafish fed the experimental diets compared with zebrafish fed the reference diet (Figures 18 and 19). Additional Menhaden oil was added to the arginine experimental diets with the goal of increasing growth performance. Addition of Menhadden oil

resulted in an increase in crude lipid level from 5 to 8% and an increase in gross energy from 4760 to 4990 cal/g (Tables 3 and 4), however, growth was similar to Experiment 1.

Feeds that made up the reference diet regimen had crude protein contents above 52% (Table 11) while crude protein of the experimental diets were between 27 and 39% (Tables 3 and 4). O'Brine et al. (2015) determined that zebrafish fed diets containing crude protein ranging from 32 to 75% showed no difference in specific growth rate. Furthermore, they demonstrated that a diet with 32% crude protein and 8% crude lipid would meet growth requirements of zebrafish (O'Brine et al., 2015).

Casein and gelatin were the primary sources of protein in the diets of Experiment 1 and 2. Nutrient requirement studies of other fish species utilized a combination of fishmeal, gluten meal, casein and gelatin in their experimental diets (Twibell et al., 2003; Zhou, et al., 2007, Cao et al., 2012; Kwasek et al., 2012; Ren, et al., 2013; Lin et al., 2013), which resulted in adequate growth performance. It is suspected that the inclusion of fishmeal and/or gluten meal could increase palatability or add some essential micronutrients that were absent in the experimental diets of the present study. Twibell et al. (2003) emphasized the importance of using both ascorbic acid and choline in experimental diets. Investigations into improving zebrafish growth performance by including fishmeal, gluten meal, choline chloride, lecithin and/or ascorbic acid to experimental diets should be performed before further nutritional research is conducted with zebrafish.

Conclusions

The current experiments are the first to determine any quantitative nutrient requirements of zebrafish. Data indicate that dietary lysine requirement for juvenile and adult zebrafish is 2.2% of dry diet and 5.6% of protein. Data regarding the arginine requirement for juvenile zebrafish were inconclusive while the arginine requirement for adult zebrafish was found to be 1.95% of dry diet and 6.5% of protein, however plasma arginine concentrations did not confirm these findings. Inconclusive results may be due to biosynthesis of arginine from proline. Antagonism between lysine and arginine was not observed in adult zebrafish fed diets containing arginine between 0.80 and 2.70%. Factorial studies utilizing feeds containing varying lysine and arginine concentrations should be conducted to determine if an antagonism exists. Using the quantitative lysine data obtained from the present study in combination with the ideal protein profile of zebrafish, quantitative requirements for all 10 EAA were estimated. These estimates should be used as a general guide to the actual requirements. Quantitative requirements for all EAA should be determined using dose-response quantitative studies, similar to the present study. Further research on macronutrient requirements for zebrafish should be conducted in order to establish a standardized diet for this important model organism.

Table 1. Temperature and water chemistry values during Experiment 1.

Temperature	27.5-28.5^{o}C
Ammonia	0 ppm
Nitrate	<60 ppm
Nitrite	<0.5 ppm
Hardness	75-150 ppm
Chlorine	0 ppm
Alkalinity	40-80 ppm
pH	6.8 - 7.6

Table 2. Temperature and water chemistry values during Experiment 2.

Temperature	26.7-28.5^{o}C
Ammonia	0 ppm
Nitrate	<60 ppm
Nitrite	0 ppm
Hardness	75-150 ppm
Chlorine	0 ppm
Alkalinity	20-80 ppm
pH	6.8 - 7.0

Table 3. Ingredient and chemical composition of test diets fed to zebrafish in Experiment 1.

	Diets (g kg^{-1} dry weight)						
Item	1	2	3	4	5	6	7
Ingredient							
Casein[1]	80	80	80	80	80	80	80
Gelatin[2]	230	230	230	230	230	230	230
Dextrin[3]	200	200	200	200	200	200	200
Alpha Cellulose[1]	218	218	218	218	218	218	218
Carboxymethyl Cellulose[1]	20	20	20	20	20	20	20
Mineral Mix[2,4]	30	30	30	30	30	30	30
Vitamin Mix[2,5]	30	30	30	30	30	30	30
Menhadden Oil[1]	140	140	140	140	140	140	140
L-lysine[2]	0	4	6	8	10	12	16
L-Glutamic Acid[1]	16	12	10	8	6	4	0
L-Histidine[1]	2	2	2	2	2	2	2
L-Threonine[1]	9	9	9	9	9	9	9

[1] Sigma Aldrich (St Louis, MO).

[2] Florida Aqua Farms Inc. (Dade City, FL).

[3] Fisher Scientific (Pittsburgh, PA).

[4] Mineral mix (values are mg kg^{-1} dry diet): Mn 703; Zn 586; Fe 469; Cu 47; Co 6; I 469; Se 5.

[5] Vitamin mix (values are mg kg^{-1} dry diet): vitamin A 0.83; vitamin D_3 0.08; vitamin E 1443; vitamin K 54.19; vitamin B_{12} 0.68; riboflavin 0.21; p-pantothenic acid 1110.93; niacin 1.29; choline 171.81; thiamine 177.42; pyridoxine 178.55; folic acid 56.17; ascorbic acid 6289.82; biotin 2.71.

[6] All values are g kg-1 dry diet unless otherwise indicated (n=2).

Table 3. (continued).

	Diets (g kg^{-1} dry weight)						
Item	1	2	3	4	5	6	7
Ingredient							
L-Valine[1]	6	6	6	6	6	6	6
L-Isoleucine[1]	4	4	4	4	4	4	4
L-Methionine[1]	5	5	5	5	5	5	5
L-Phenylalanine[1]	6	6	6	6	6	6	6
L-Tryptophan[1]	4	4	4	4	4	4	4
Chemical composition[6]							
Crude protein	37.6	39.0	38.5	38.4	38.5	38.5	39.1
Moisture	6.5	6.4	6.7	7.7	7.0	8.0	6.4
Crude Fat	4.8	5.2	5.0	5.2	5.3	5.2	5.2
Crude Fiber	12.1	11.7	12.4	12.0	11.4	14.0	12.4
Ash	3.4	3.6	3.6	3.5	3.5	3.4	3.6
Gross Energy (cal/g) (n=3)	4832	4795	4724	4712	4745	4722	4808

[1] Sigma Aldrich (St Louis, MO).

[2] Florida Aqua Farms Inc. (Dade City, FL).

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[5] Vitamin mix (values are mg kg^{-1} dry diet): vitamin A 0.83; vitamin D_3 0.08; vitamin E 1443; vitamin K 54.19; vitamin B_{12} 0.68; riboflavin 0.21; p-pantothenic acid 1110.93; niacin 1.29; choline 171.81; thiamine 177.42; pyridoxine 178.55; folic acid 56.17; ascorbic acid 6289.82; biotin 2.71.

[6] All values are g kg-1 dry diet unless otherwise indicated (n=2).

Table 3. (continued).

	Diets (g kg^{-1} dry weight)						
Item	1	2	3	4	5	6	7
Chemical composition[6]							
Lysine	1.42	1.73	1.87	2.01	2.16	2.26	2.57
Arginine	1.94	2.11	2.2	2.04	2.14	2.13	2.08
Histidine	0.58	0.58	0.58	0.58	0.58	0.56	0.56
Isoleucine	1.14	1.2	1.21	1.17	1.19	1.15	1.16
Leucine	1.43	1.57	1.57	1.53	1.58	1.49	1.49
Methionine	0.87	0.81	0.88	0.81	0.86	0.77	0.8
Phenylalanine	1.42	1.54	1.58	1.54	1.5	1.46	1.47
Threonine	1.56	1.64	1.65	1.59	1.58	1.64	1.57
Tryptophan	0.42	0.46	0.44	0.44	0.44	0.44	0.44
Valine	1.66	1.67	1.64	1.66	1.63	1.65	1.6

[1] Sigma Aldrich (St Louis, MO).

[2] Florida Aqua Farms Inc. (Dade City, FL).

[3] Fisher Scientific (Pittsburgh, PA).

[4] Mineral mix (values are mg kg^{-1} dry diet): Mn 703; Zn 586; Fe 469; Cu 47; Co 6; I 469; Se 5.

[5] Vitamin mix (values are mg kg^{-1} dry diet): vitamin A 0.83; vitamin D_3 0.08; vitamin E 1443; vitamin K 54.19; vitamin B_{12} 0.68; riboflavin 0.21; p-pantothenic acid 1110.93; niacin 1.29; choline 171.81; thiamine 177.42; pyridoxine 178.55; folic acid 56.17; ascorbic acid 6289.82; biotin 2.71.

[6] All values are g kg-1 dry diet unless otherwise indicated (n=2).

Table 4. Ingredient and chemical composition of test diets fed to zebrafish in Experiment 2.

	Diets (g kg^{-1} dry weight)						
Item	1	2	3	4	5	6	7
Ingredient							
Casein[1]	270	270	270	270	270	270	270
Corn Starch[1]	100	100	100	100	100	100	100
Dextrin[3]	100	100	100	100	100	100	100
Alpha Cellulose[1]	204	204	204	204	204	204	204
Carboxymethyl Cellulose[1]	20	20	20	20	20	20	20
Mineral Mix[2,4]	30	30	30	30	30	30	30
Vitamin Mix[2,5]	30	30	30	30	30	30	30
Menhadden Oil[1]	200	200	200	200	200	200	200
L-arginine[1]	0	4	8	10	14	20	25
L-Glutamic Acid[1]	32	28	24	22	18	12	7
L-Lysine[2]	1	1	1	1	1	1	1
L-Threonine[1]	6	6	6	6	6	6	6

[1] Sigma Aldrich (St Louis, MO).

[2] Florida Aqua Farms Inc. (Dade City, FL).

[3] Fisher Scientific (Pittsburgh, PA).

[4] Mineral mix (values are mg kg^{-1} dry diet): Mn 703; Zn 586; Fe 469; Cu 47; Co 6; I 469; Se 5.

[5] Vitamin mix (values are mg kg^{-1} dry diet): vitamin A 0.83; vitamin D_3 0.08; vitamin E 1443; vitamin K 54.19; vitamin B_{12} 0.68; riboflavin 0.21; p-pantothenic acid 1110.93; niacin 1.29; choline 171.81; thiamine 177.42; pyridoxine 178.55; folic acid 56.17; ascorbic acid 6289.82; biotin 2.71.

[6] All values are g kg-1 dry diet unless otherwise indicated (n=2).

Table 4. (continued).

	Diets (g kg^{-1} dry weight)						
Item	1	2	3	4	5	6	7
Ingredient							
L-Methionine[1]	2	2	2	2	2	2	2
L-Phenylalanine[1]	2	2	2	2	2	2	2
L-Tryptophan[1]	3	3	3	3	3	3	3
Chemical composition[6]							
Crude protein	27.8	29.1	30.3	29.8	30.9	31.2	31.9
Moisture	8.5	6.6	6.3	6.6	6.3	8.4	8.0
Crude Fat	8.0	8.3	8.1	8.0	8.1	8.8	8.6
Crude Fiber	11.3	10.1	9.6	8.6	9.8	9.7	8.9
Ash	3.5	3.7	3.6	3.7	3.7	3.5	3.5
Gross Energy (cal/g) (n=3)	4921	5077	5031	4967	5062	4910	4969
Lysine	2.08	2.08	2.09	2.09	2.07	2.16	2.21
Arginine	0.8	1.08	1.43	1.56	1.88	2.37	2.7

[1] Sigma Aldrich (St Louis, MO).

[2] Florida Aqua Farms Inc. (Dade City, FL).

[3] Fisher Scientific (Pittsburgh, PA).

[4] Mineral mix (values are mg kg^{-1} dry diet): Mn 703; Zn 586; Fe 469; Cu 47; Co 6; I 469; Se 5.

[5] Vitamin mix (values are mg kg^{-1} dry diet): vitamin A 0.83; vitamin D_3 0.08; vitamin E 1443; vitamin K 54.19; vitamin B_{12} 0.68; riboflavin 0.21; p-pantothenic acid 1110.93; niacin 1.29; choline 171.81; thiamine 177.42; pyridoxine 178.55; folic acid 56.17; ascorbic acid 6289.82; biotin 2.71.

[6] All values are g kg-1 dry diet unless otherwise indicated (n=2).

Table 4. (continued).

	Diets (g kg^{-1} dry weight)						
Item	1	2	3	4	5	6	7
Chemical composition[6]							
Histidine	0.72	0.7	0.72	0.71	0.71	0.75	0.74
Isoleucine	1.37	1.36	1.36	1.33	1.36	1.32	1.39
Leucine	2.48	2.45	2.45	2.46	2.46	2.45	2.51
Methionine	0.79	0.8	0.81	0.82	0.82	0.84	0.88
Phenylalanine	1.53	1.53	1.54	1.51	1.54	1.54	1.61
Threonine	1.58	1.57	1.62	1.6	1.58	1.69	1.74
Tryptophan	0.51	0.53	0.56	0.51	0.52	0.51	0.52
Valine	1.65	1.63	1.62	1.62	1.61	1.66	1.73

[1] Sigma Aldrich (St Louis, MO).

[2] Florida Aqua Farms Inc. (Dade City, FL).

[3] Fisher Scientific (Pittsburgh, PA).

[4] Mineral mix (values are mg kg^{-1} dry diet): Mn 703; Zn 586; Fe 469; Cu 47; Co 6; I 469; Se 5.

[5] Vitamin mix (values are mg kg^{-1} dry diet): vitamin A 0.83; vitamin D_3 0.08; vitamin E 1443; vitamin K 54.19; vitamin B_{12} 0.68; riboflavin 0.21; p-pantothenic acid 1110.93; niacin 1.29; choline 171.81; thiamine 177.42; pyridoxine 178.55; folic acid 56.17; ascorbic acid 6289.82; biotin 2.71.

[6] All values are g kg-1 dry diet unless otherwise indicated (n=2).

Table 5. Survival, mean final weight and mean final length* of juvenile zebrafish fed diets of varying lysine concentration in Experiment 1.

Lysine level (% of diet)	Survival (%)	Final weight (mg)	Final length (mm)
1.42	100	40.7 ± 3.0	16.6 ± 0.7[ab]
1.73	83	33.6 ± 3.1	15.2 ± 0.3[a]
1.87	97	43.1 ± 14.2	16.3 ± 1.0[ab]
2.01	93	49.5 ± 7.3	16.9 ± 0.6[ab]
2.16	97	48.8 ± 9.6	18.1 ± 0.4[b]
2.26	100	47.9 ± 2.6	17.6 ± 0.3[b]
2.57	93	41.5 ± 7.5	16.5 ± 1.0[ab]

* Values are means of triplicate groups ± SD.

[a,b] Means within a column with uncommon superscripts differ ($P < 0.05$).

Table 6. Survival and mean embryo production* of adult zebrafish fed diets of varying lysine concentration in Experiment 1.

Lysine level (% of diet)	Survival (%)	Embryo production
1.42	100	209.3 ± 46.5^{ab}
1.73	93	149.7 ± 128.6^{ab}
1.87	90	184.7 ± 81.1^{ab}
2.01	100	68.3 ± 75.9^{a}
2.16	93	436.3 ± 236.9^{b}
2.26	87	79.7 ± 49.0^{a}
2.57	100	80.0 ± 116.0^{a}

* Embryo production values are means of triplicate groups ± SD.

[a,b] Means within a column with uncommon superscripts differ ($P < 0.05$).

Table 7. Plasma lysine concentrations of fish fed diets of varying lysine concentrations in Experiment 1.

Dietary lysine (% of diet)	Plasma lysine (uM)
1.42	139.9
1.73	83.3
1.87	126.9
2.01	127.5
2.16	124.9
2.26	146.7
2.57	146.8

Table 8. Survival, mean final weight and mean final length* of juvenile zebrafish fed diets of varying arginine concentrations in Experiment 2.

Arginine level (% of diet)	Survival (%)	Final weight (mg)	Final length (mm)
0.80	100	41.9 ± 5.2	17.0 ± 0.5
1.08	93	39.3 ± 4.7	16.8 ± 0.7
1.43	97	35.8 ± 4.2	16.1 ± 1.3
1.56	100	37.9 ± 2.3	16.5 ± 0.6
1.88	100	32.1 ± 6.5	16.1 ± 0.9
2.37	93	29.0 ± 6.7	15.5 ± 1.3
2.70	93	43.3 ± 8.6	17.2 ± 1.4

* Values are means of triplicate groups ± SD.

[a,b] Means within a column with uncommon superscripts differ ($P < 0.05$).

Table 9. Survival, mean embryo production and plasma arginine concentration* of adult zebrafish fed diets of varying arginine concentrations in Experiment 2.

Arginine level	Survival (%)	Embryo production	Plasma arginine (uM)
0.80	93	321.3 ± 153.9	92.1 ± 9.1
1.08	100	273.0 ± 131.3	85.8 ± 16.5
1.43	97	234.3 ± 106.3	148.7 ± 67.1
1.56	100	229.7 ± 206.9	98.5 ± 61.1
1.88	93	338.0 ± 447.2	91.5 ± 11.3
2.37	90	154.0 ± 159.8	94.2 ± 28.4
2.70	90	58.3 ± 101.0	97.2 ± 44.1

* Embryo production and plasma arginine values are means of triplicate groups ± SD.

[a,b] Means within a column with uncommon superscripts differ ($P < 0.05$).

Table 10. Estimated essential amino acid requirements for zebrafish based upon the ideal protein profile.

Essential Amino Acid	Estimated Requirement (% of dry diet)
Lys	2.2
Arg	1.7
His	0.7
Ile	1.2
Leu	2.1
Met + Cys	1.0
Phe + Tyr	2.1
Thr	1.2
Trp	Not determined
Val	1.4

Table 11. Approximate composition of reference diet feeds.

Chemical composition (% dry matter)	Decpsulated Brine Shrimp[1]	Zebrafish Select Diet[2]
Crude protein	53.5	> 52.0
Moisture	8.3	
Crude Fat	2.3	16.0
Ash	9.0	< 12.0

[1] Brine Shrimp Direct, Ogden, UT

[2] Aquaneering, San Diego, CA

Figure 1. Broken-line regression analysis of mean final weight for juvenile zebrafish fed diets of varying lysine concentrations in Experiment 1.

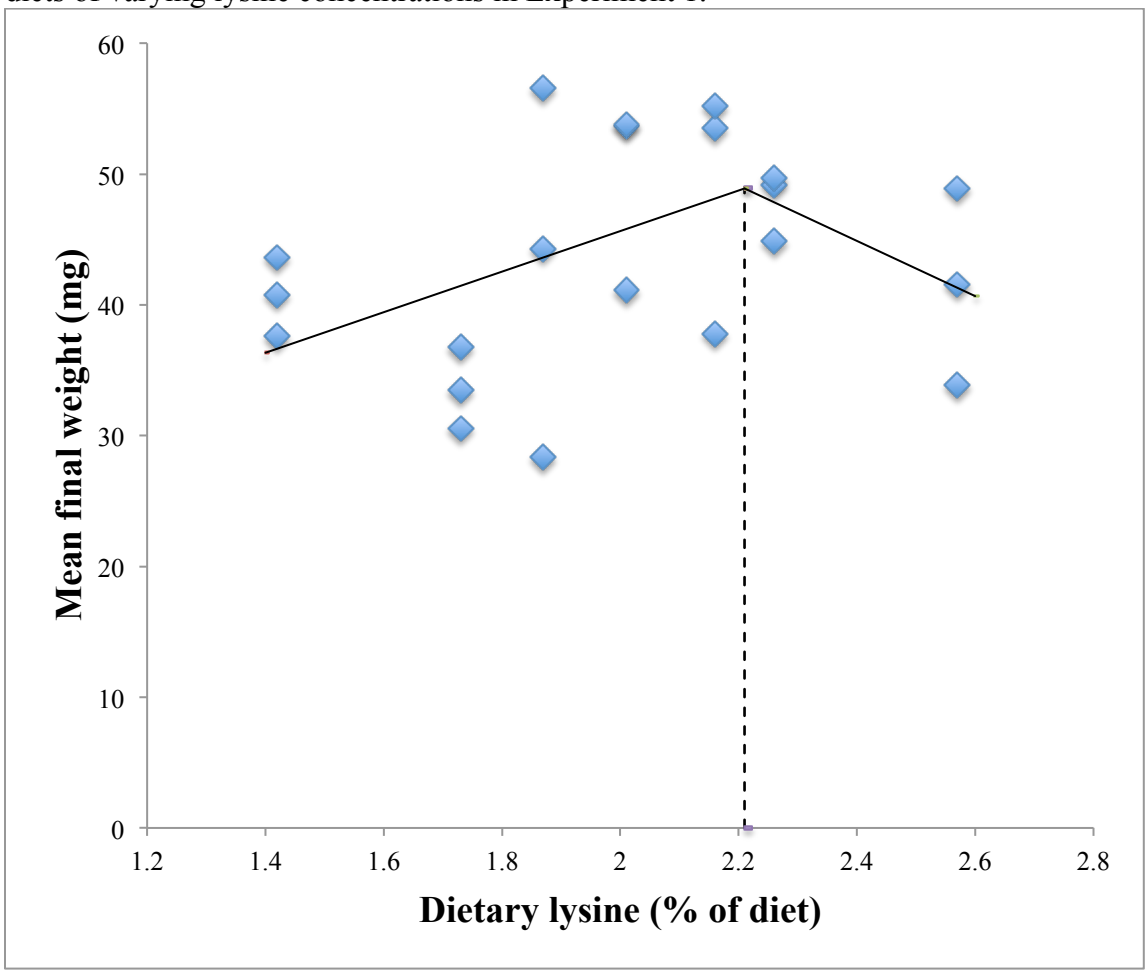


Figure 2. Quadratic regression analysis of mean final weight for juvenile zebrafish fed diets of varying lysine concentrations in Experiment 1.

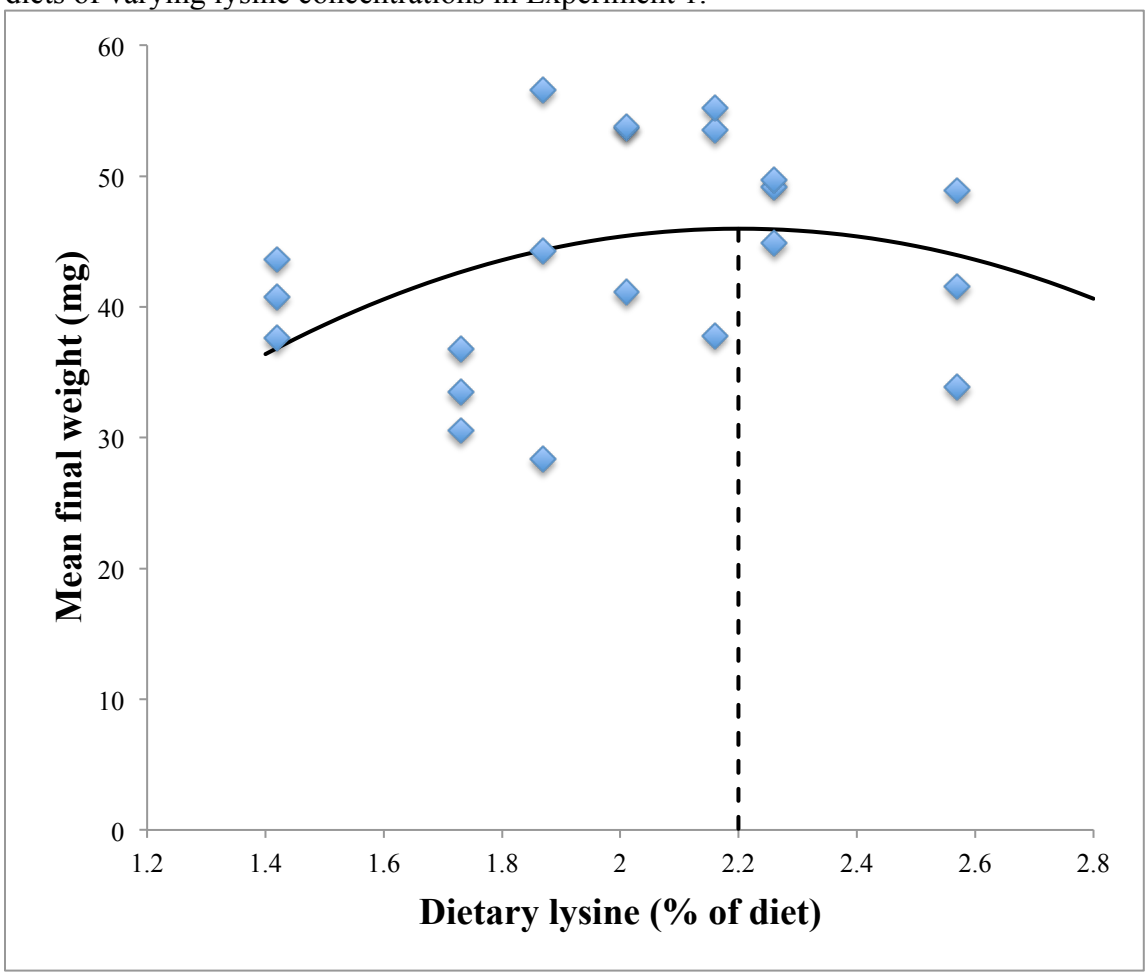


Figure 3. Quadratic regression analysis of mean final length for juvenile zebrafish fed diets of varying lysine concentrations in Experiment 1.

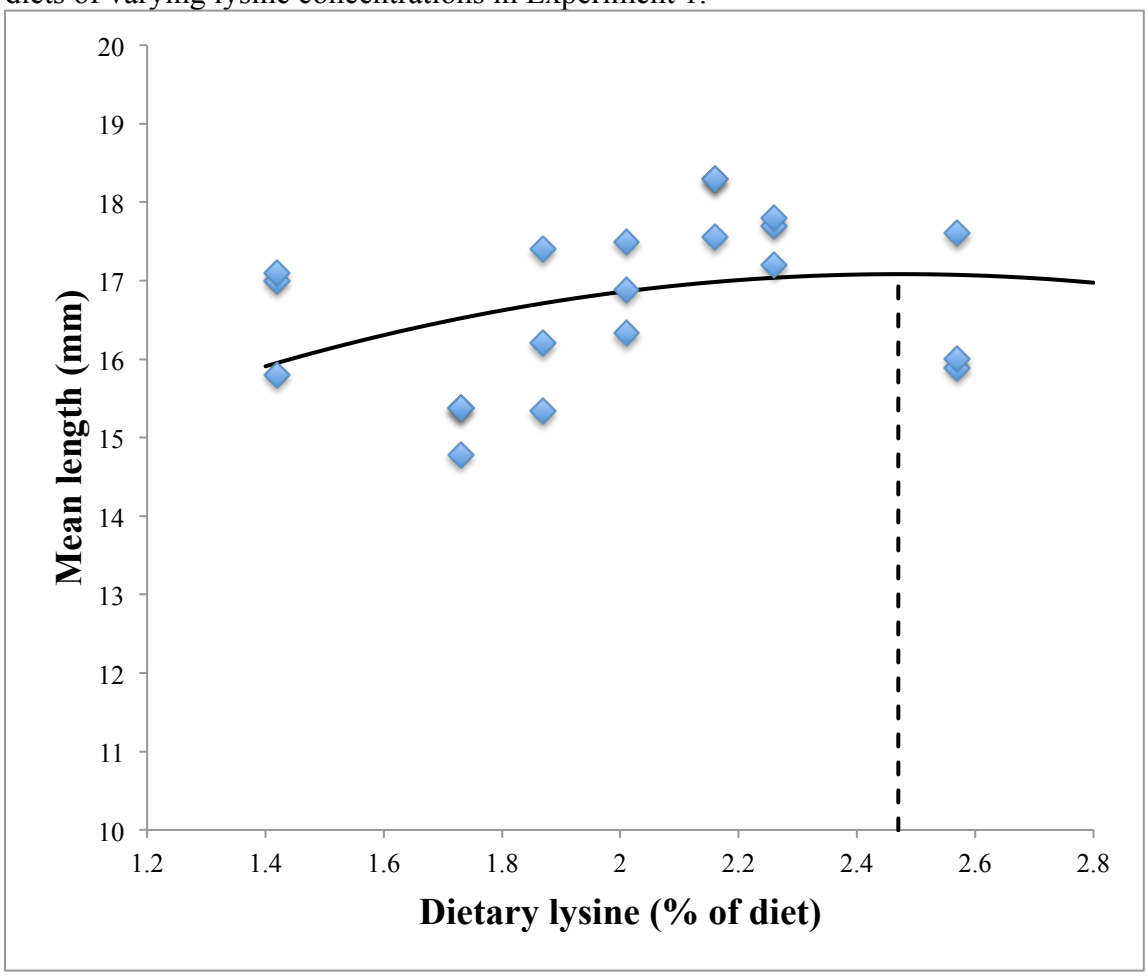


Figure 4. Broken-line regression analysis of embryo production for adult zebrafish fed diets of varying lysine concentrations in Experiment 1.

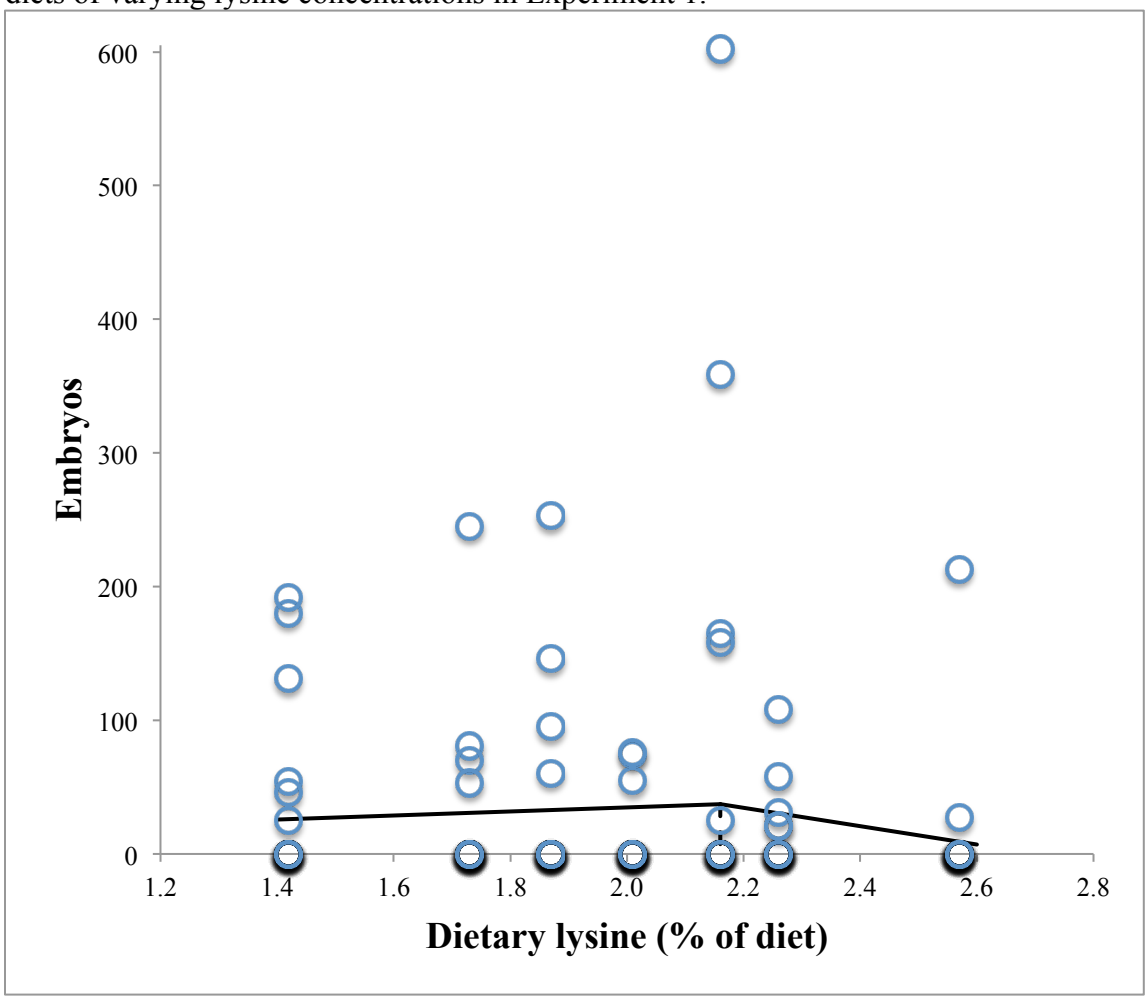


Figure 5. Quadratic regression analysis of embryo production for adult zebrafish fed diets of varying lysine conentrations in Experiment 1..

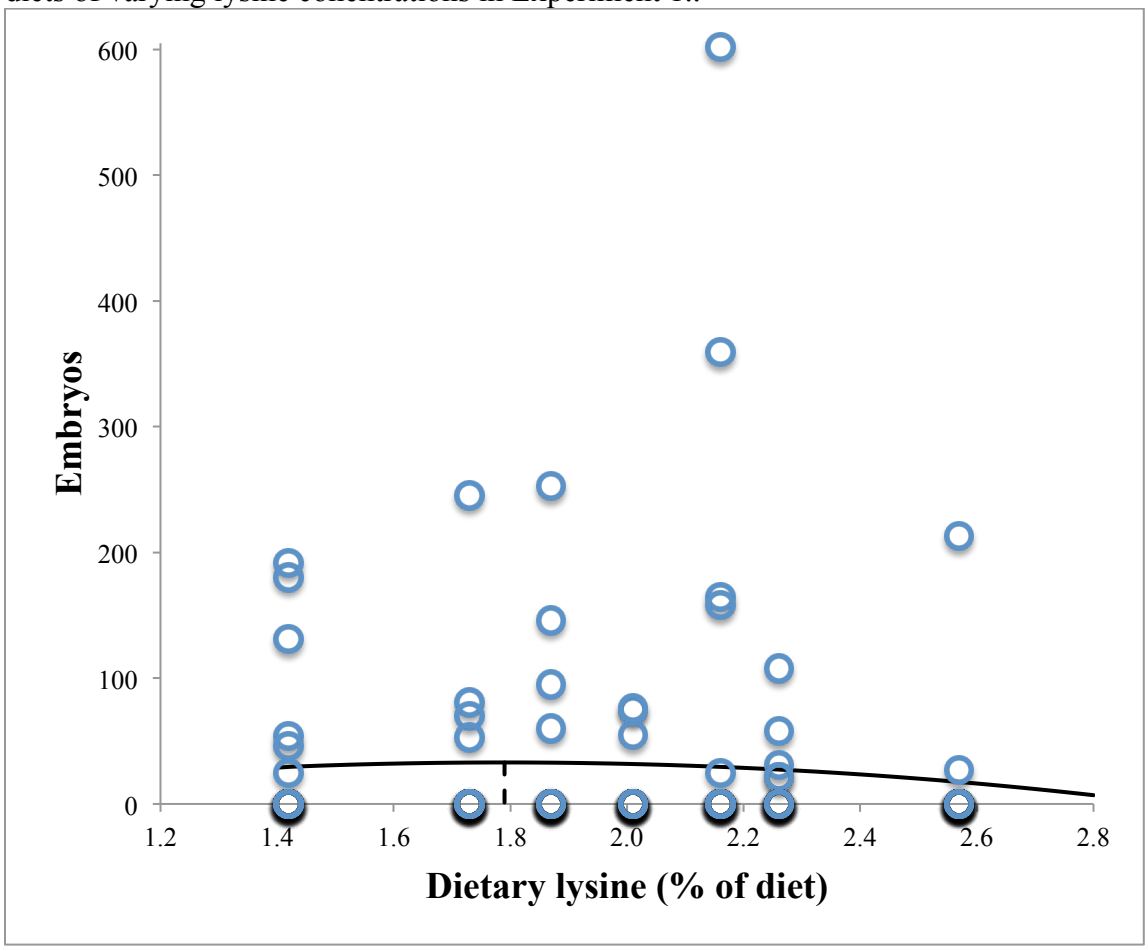


Figure 6. Quadratic regression analysis of final weights for jubenile zebrafish fed diets of varying arginine concentrations in Experiment 2.

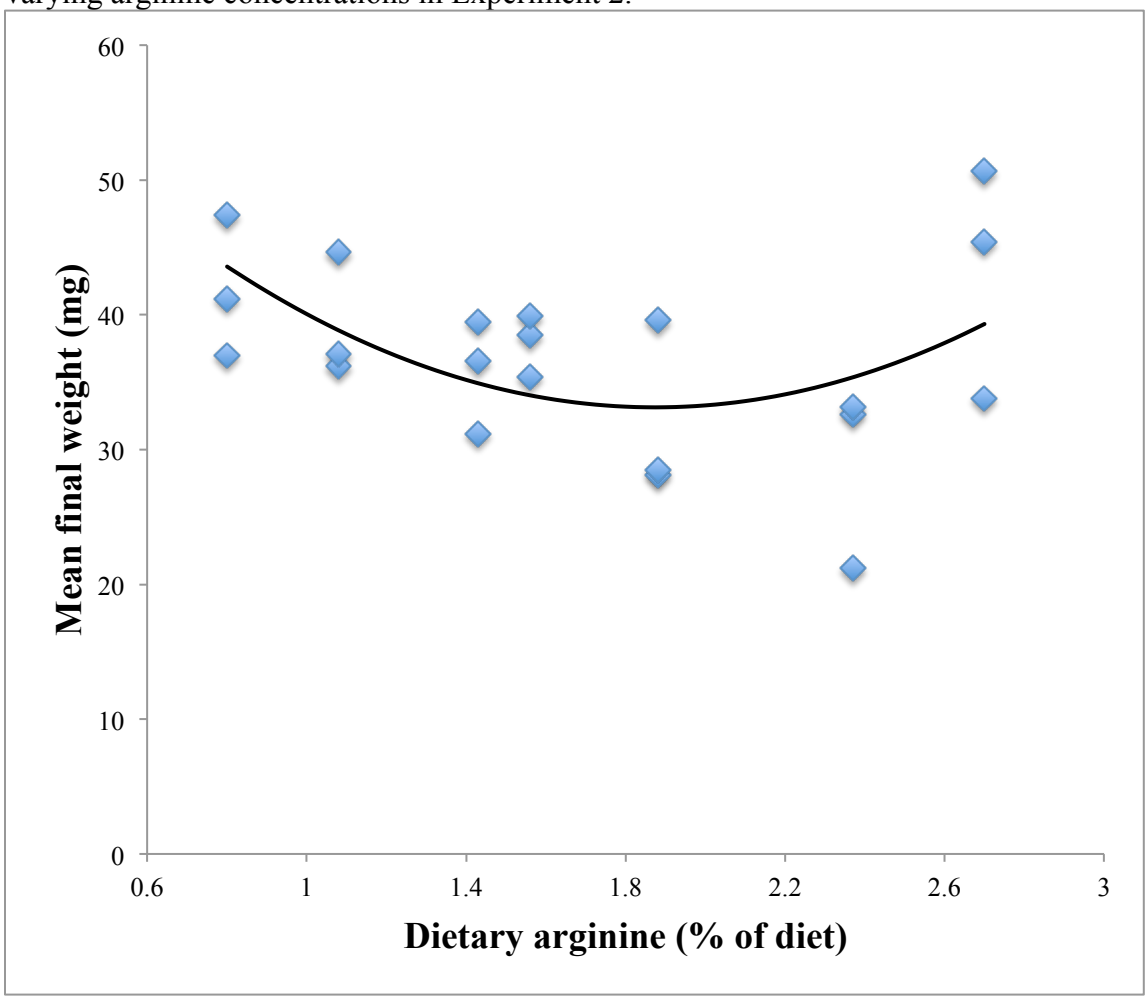


Figure 7. Quadratic regression analysis of final lengths for juvenile zebrafish fed diets of varying arginine concentrations in Experiment 2.

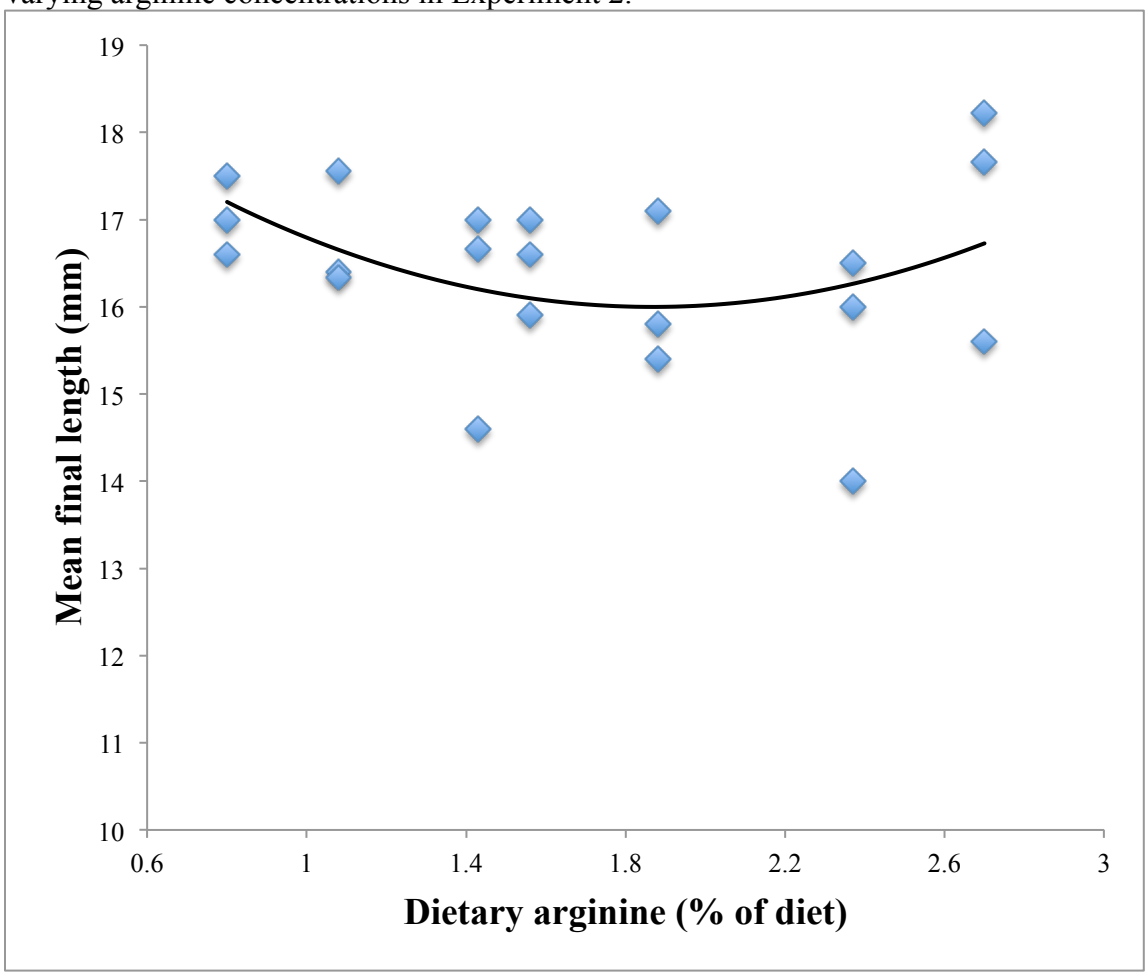


Figure 8. Broken-line regression analysis of embryo production for adult zebrafish fed diets of varying arginine concentrations in Experiment 2.

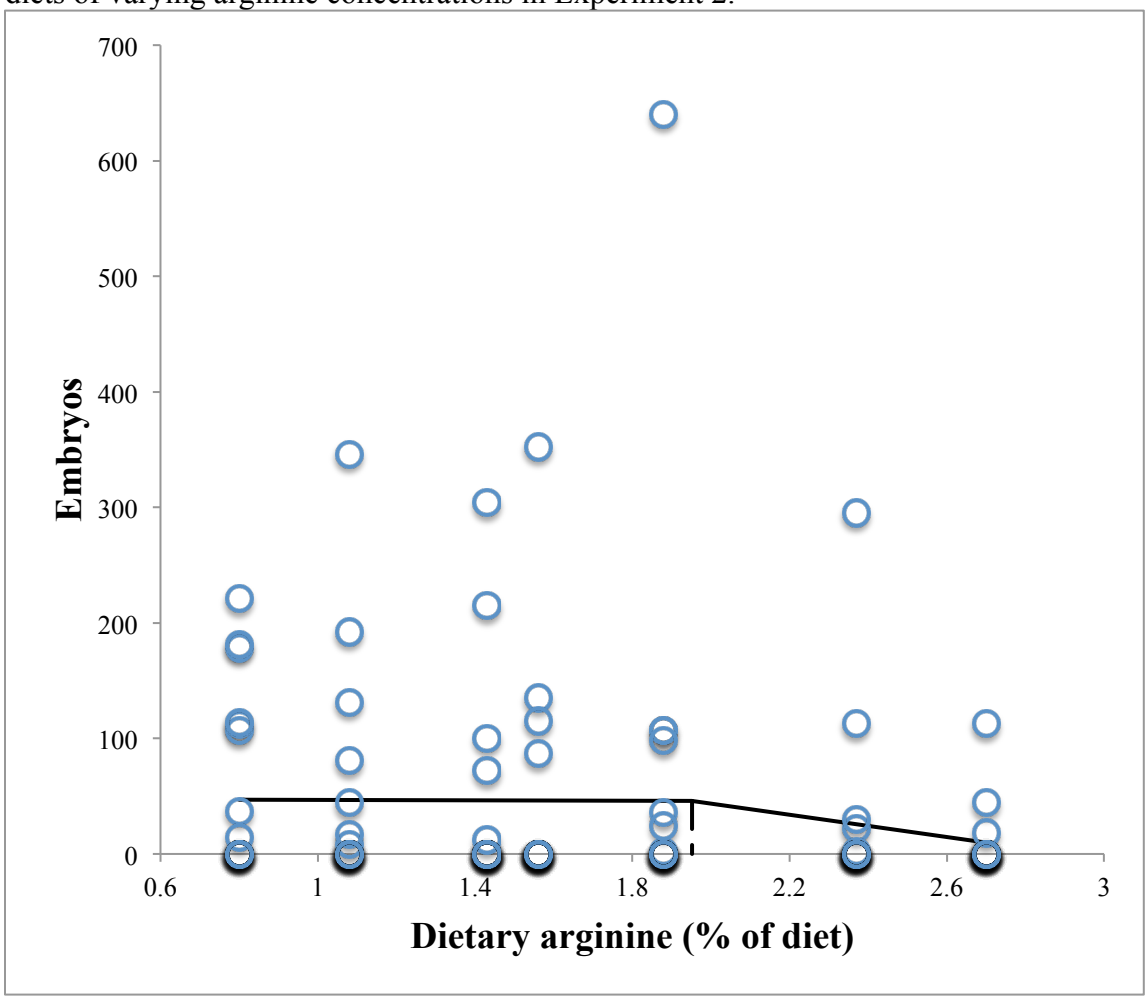


Figure 9. Quadratic regression analysis of embryo production for adult zebrafish fed diets of varying arginine concentrations in Experiment 2.

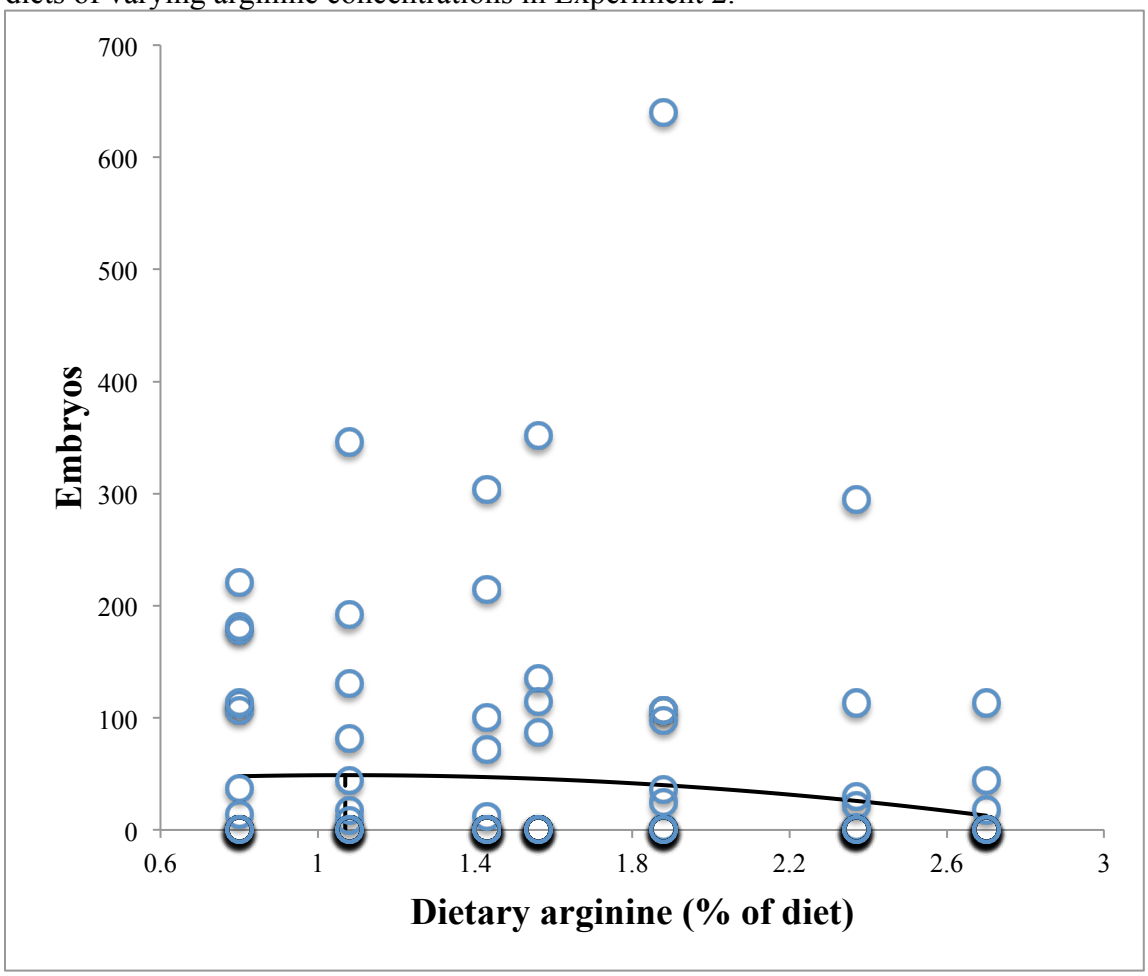


Figure 10. Broken-line regression analysis of plasma arginine concentrations for adult zebrafish fed diets of varying arginine concentrations in Experiment 2.

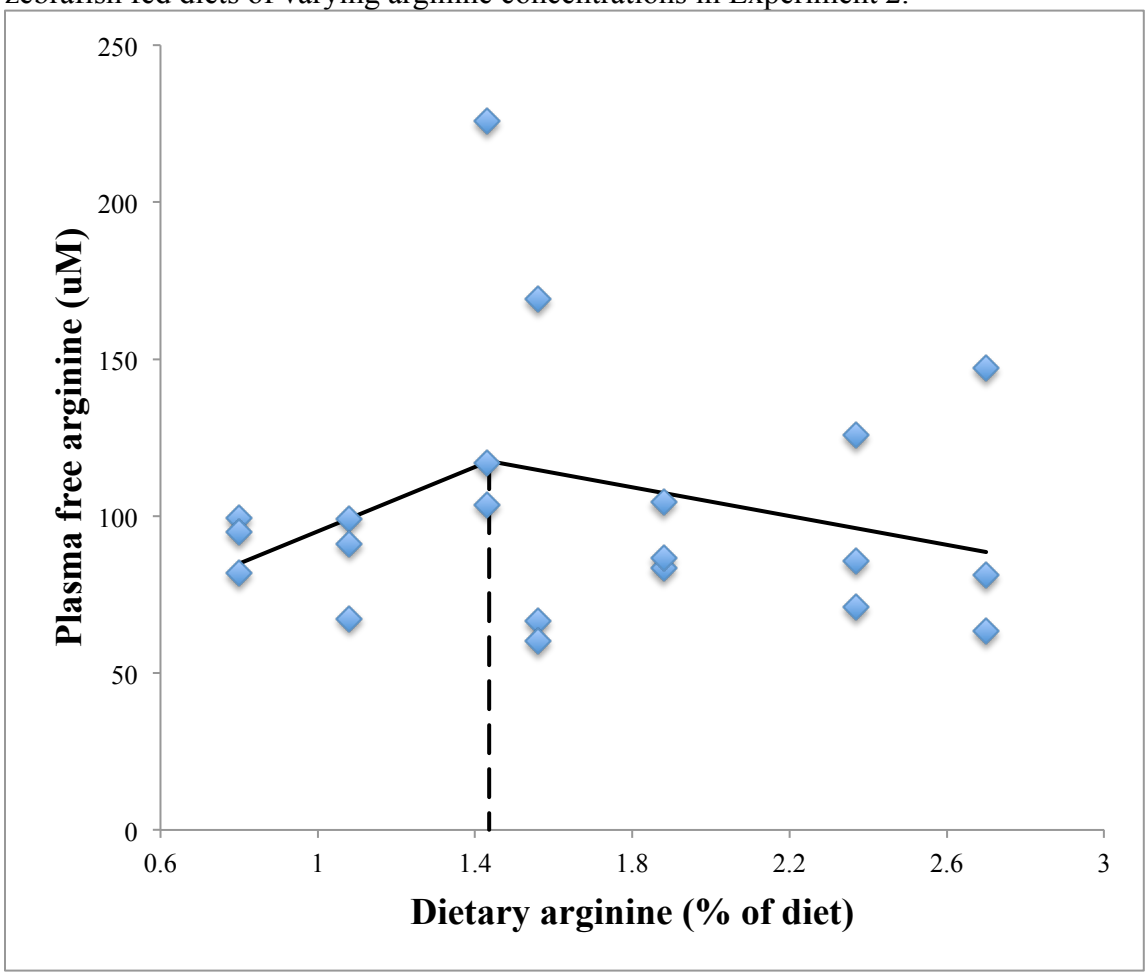


Figure 11. Quadratic regression analysis of plasma arginine concentrations for adult zebrafish fed diets of varying arginine concentrations in Experiment 2.

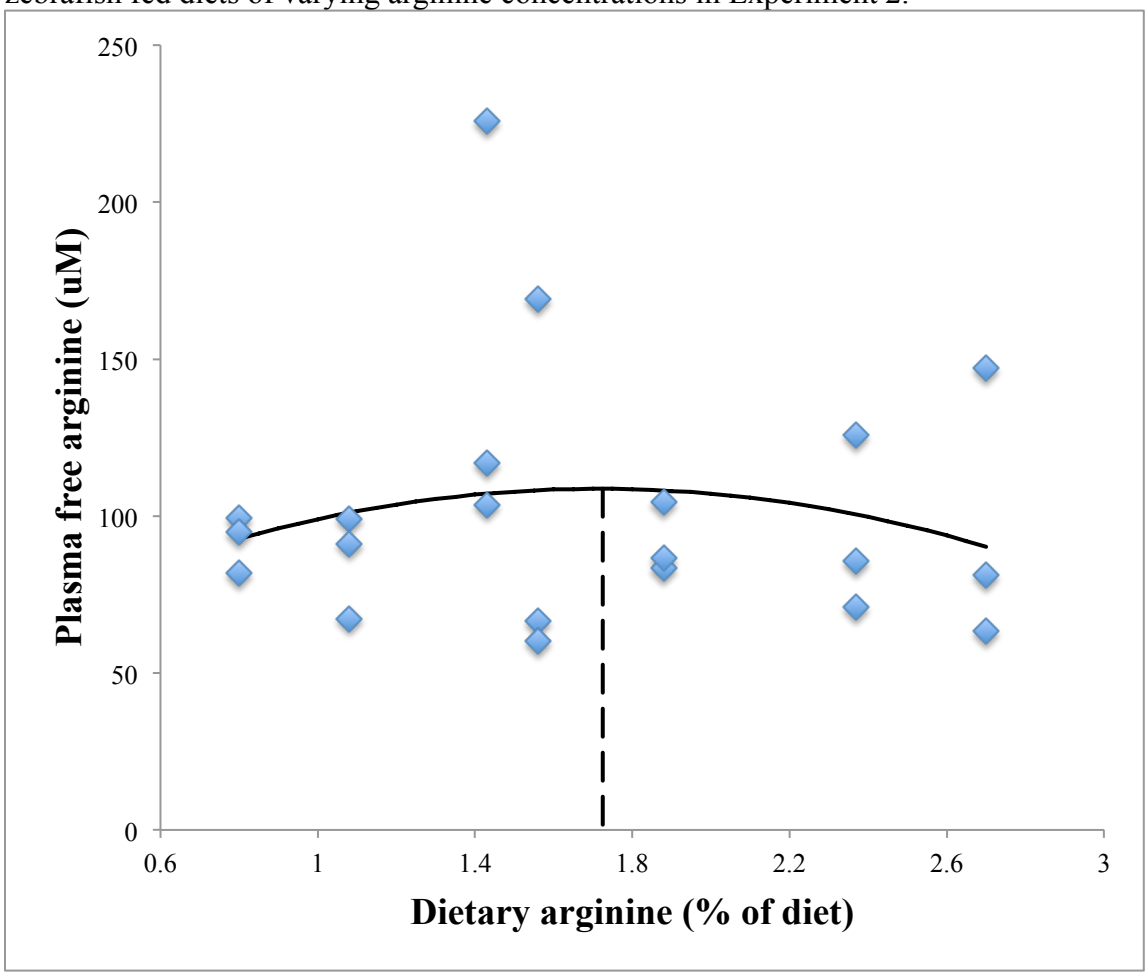


Figure 12. Mean final weights for juvenile zebrafish fed reference diet and diets of varying lysine concentrations in Experiment 1.

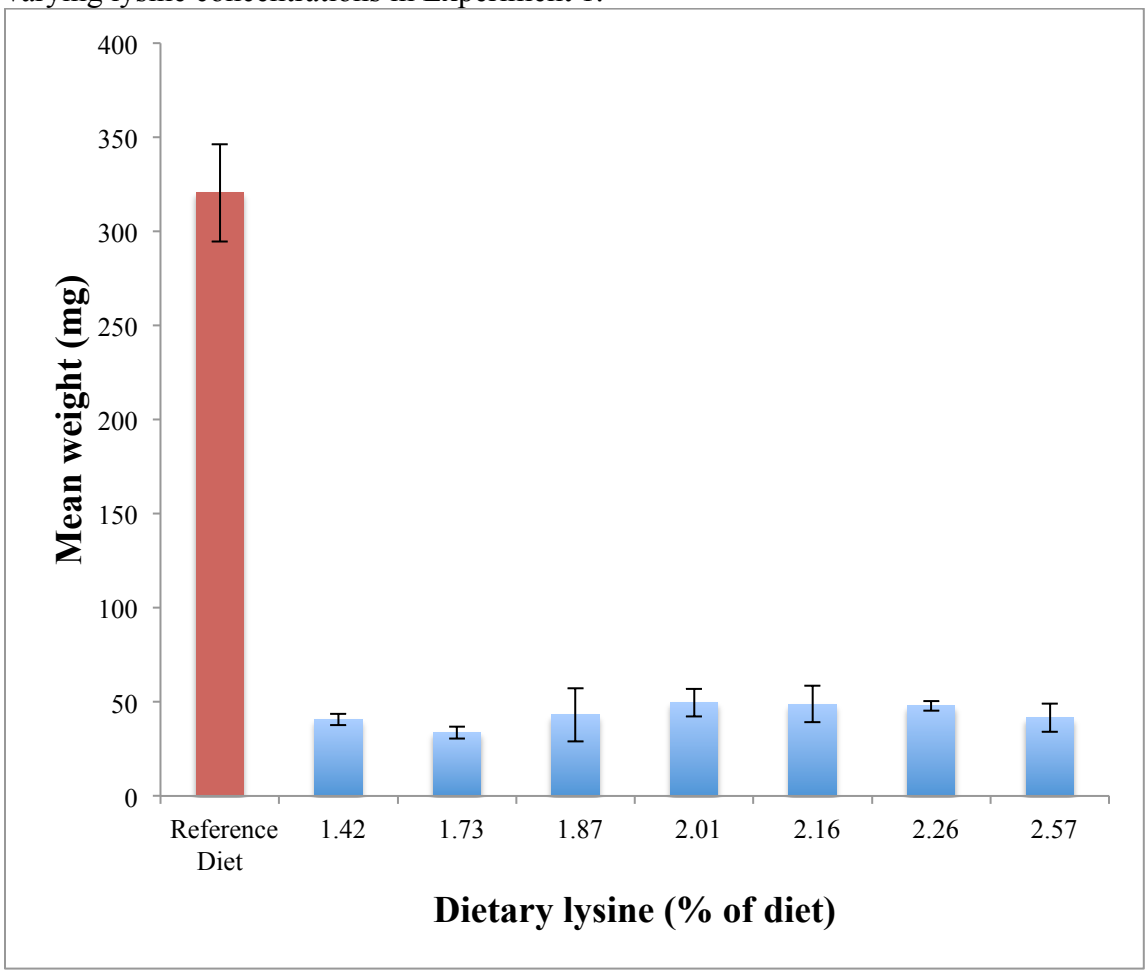


Figure 13. Mean lengths for juvenile zebrafish fed reference diet and diets of varying lysine concentrations in Experiment 1.

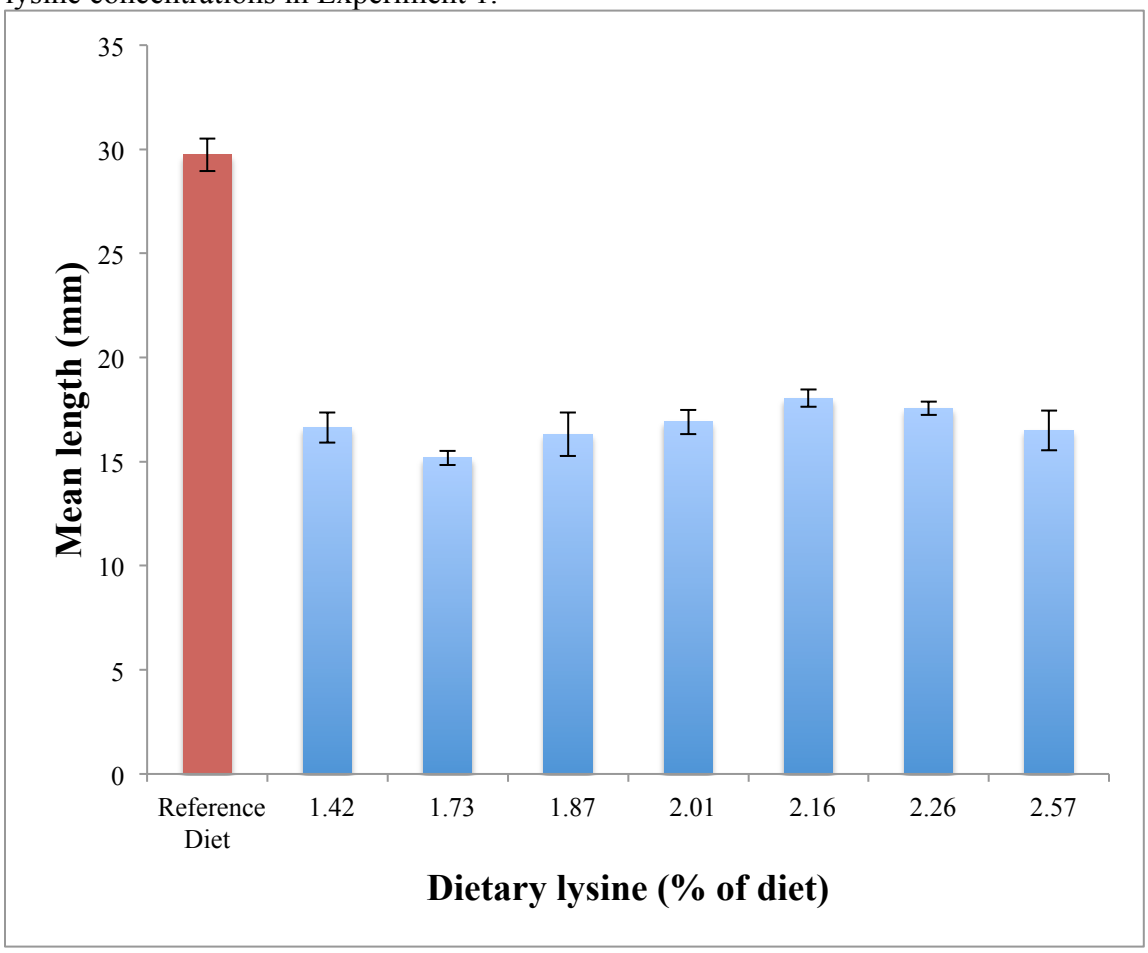


Figure 14. Plasma lysine concentrations of adult zebrafish fed reference diet and diets of varying lysine concentrations in Experiment 1.

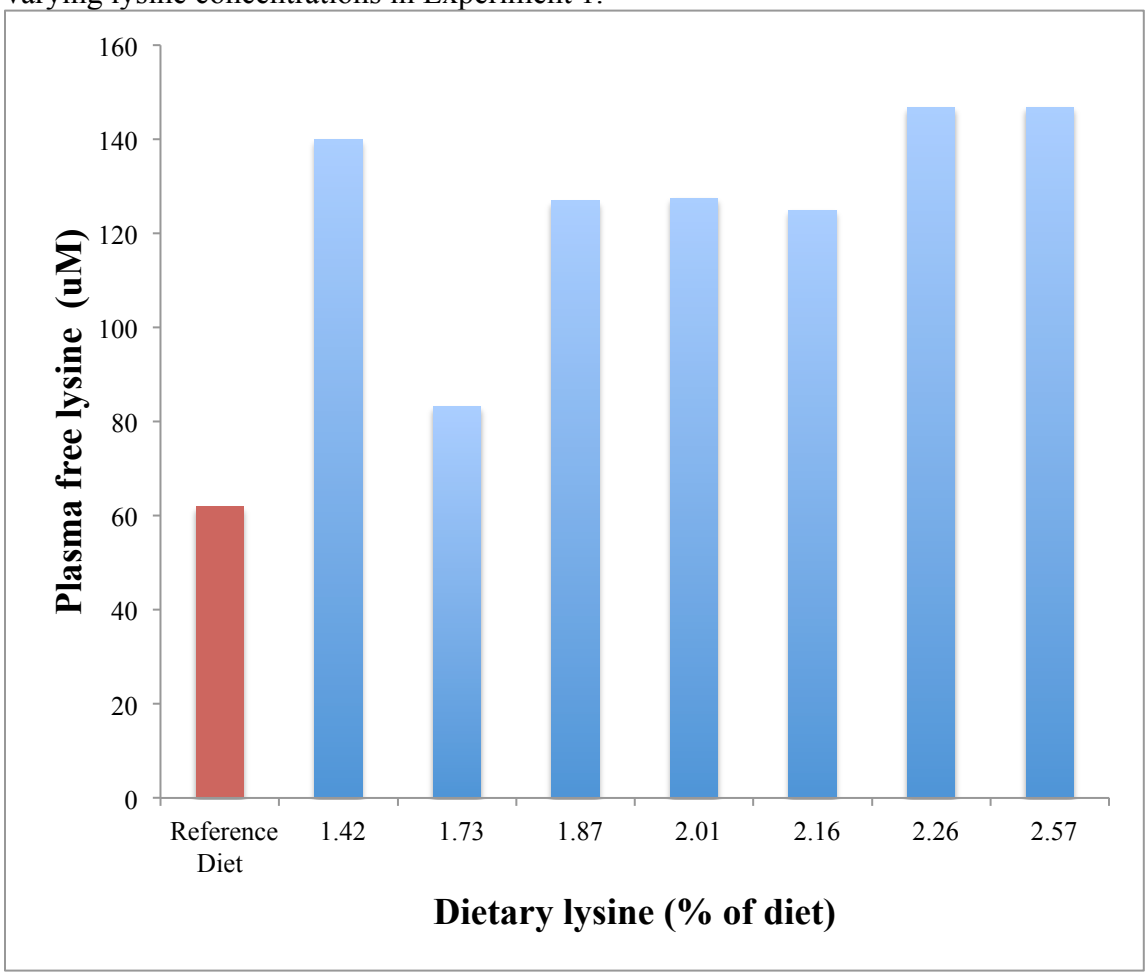


Figure 15. Plasma citrulline concentrations of adult zebrafish fed diets of varying arginine concentrations in Experiment 2.

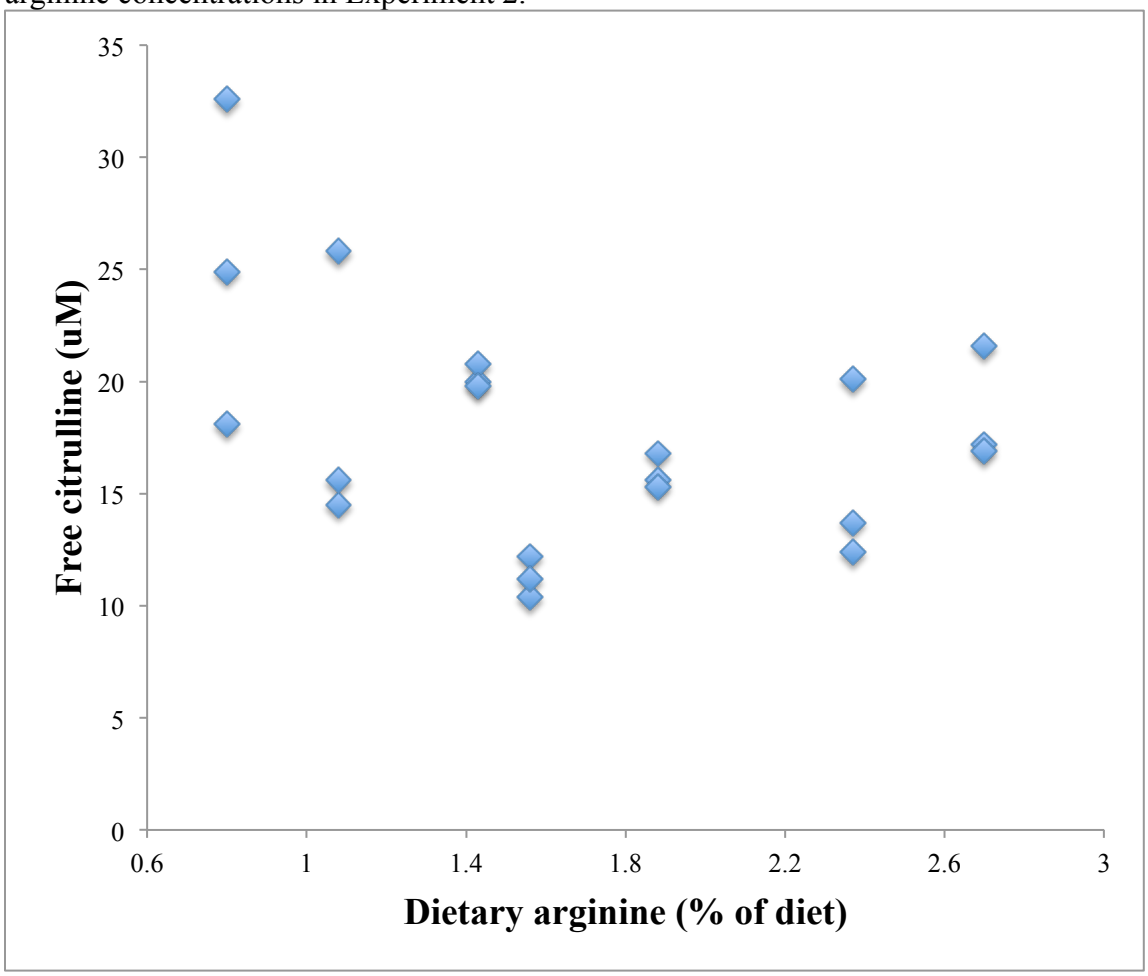


Figure 16. Plasma proline concentrations of adult zebrafish fed diets of varying arginine concentrations in Experiment 2.

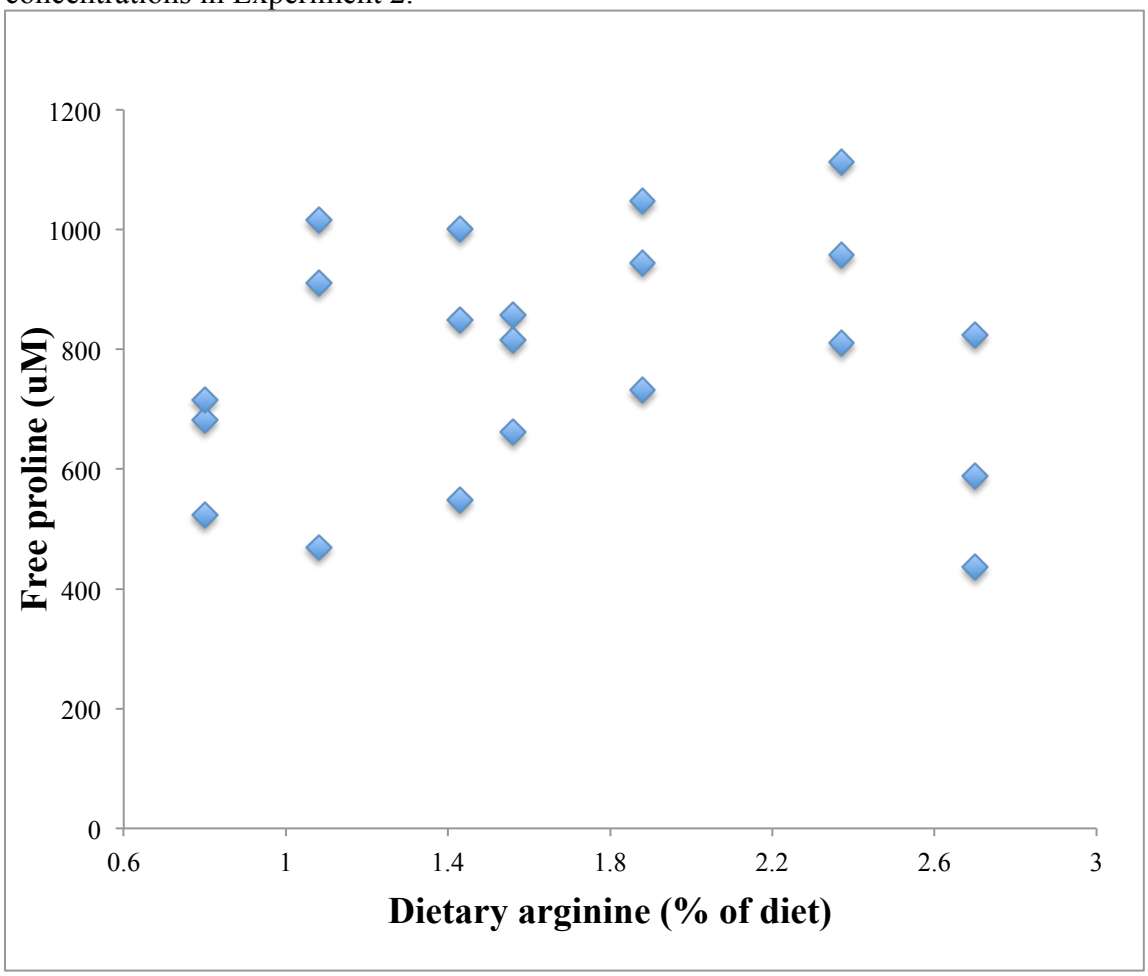


Figure 17. Plasma lysine concentrations of adult zebrafish fed diets of varying arginine concentrations in Experiment 2.

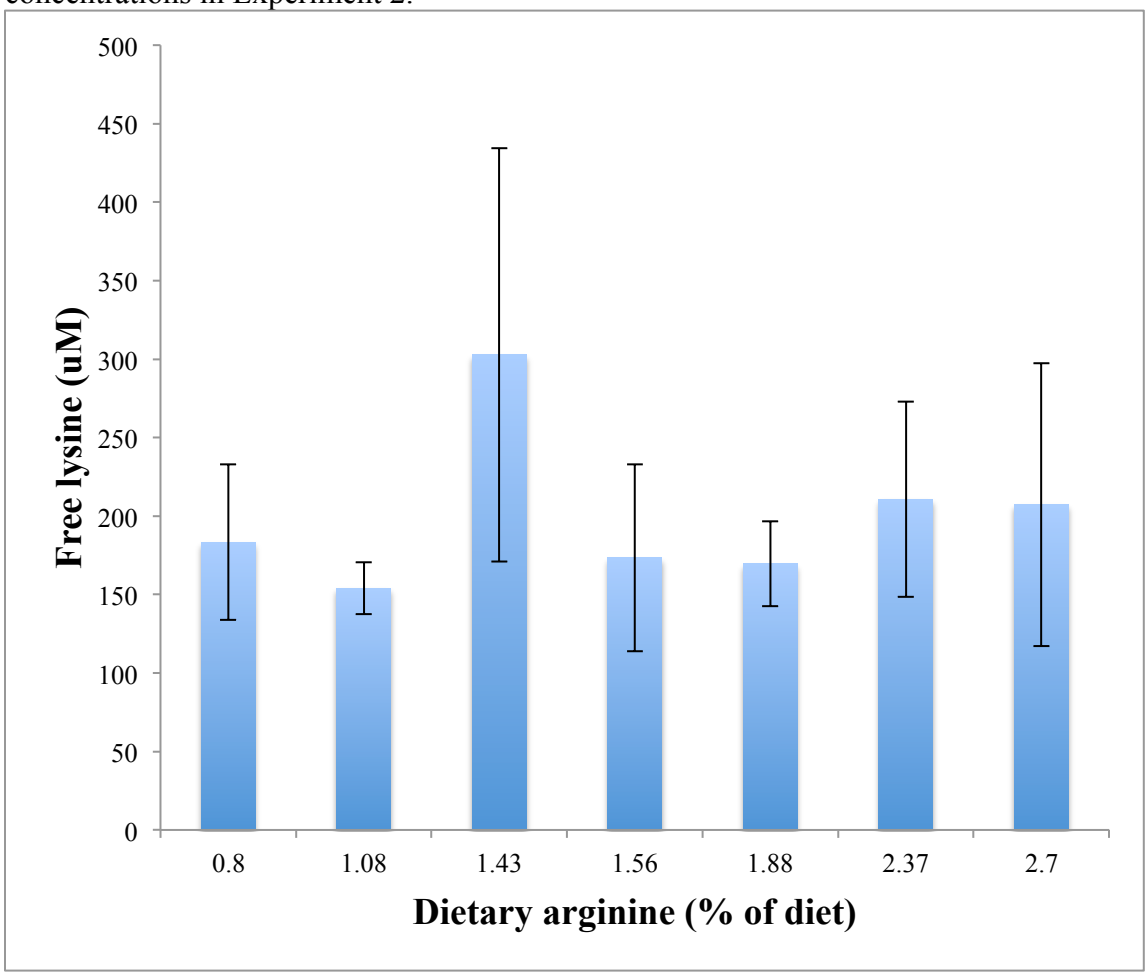


Figure 18. Mean weights for juvenile zebrafish fed reference diet and diets of varying arginine concentrations in Experiment 2.

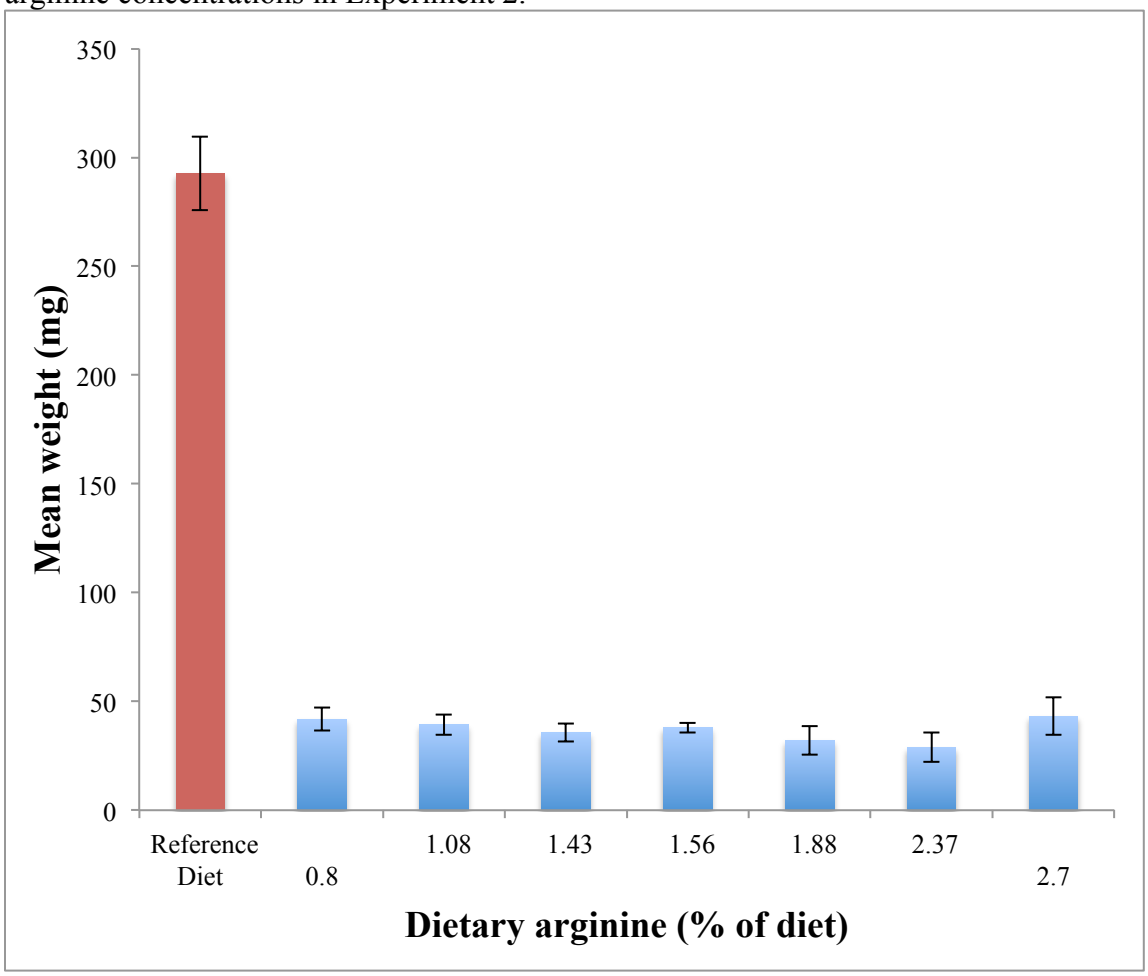
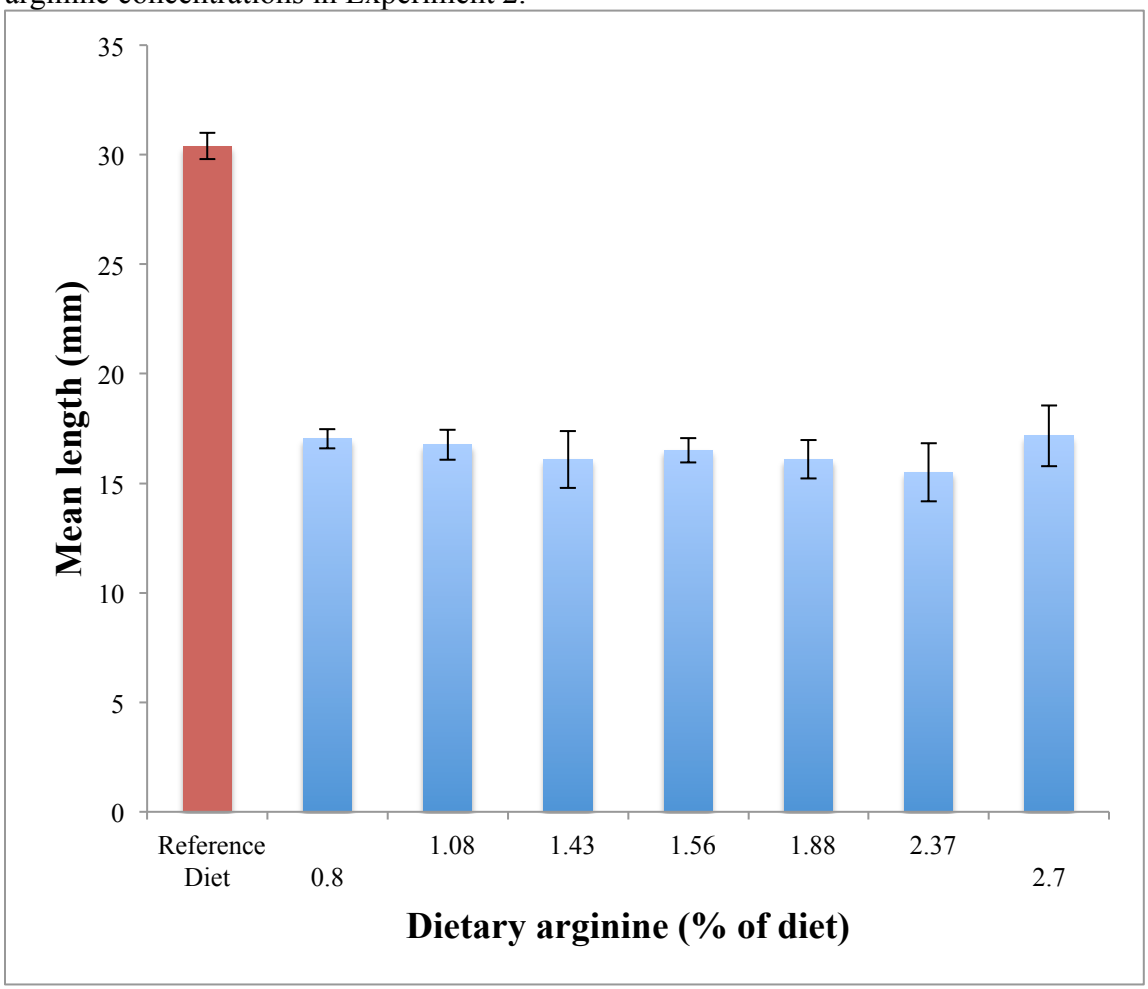


Figure 19. Mean lengths for juvenile zebrafish fed reference diet and diets of varying arginine concentrations in Experiment 2.



REFERENCES

Ahmed, I., & Khan, M. A. (2004). Dietary lysine requirement of fingerling indian major carp, cirrhinus mrigala (hamilton). *Aquaculture, 235*(1), 499-511.

Alam, M., Teshima, S., Ishikawa, M., & Koshio, S. (2002). Effects of dietary arginine and lysine levels on growth performance and biochemical parameters of juvenile Japanese flounder paralichthys olivaceus. *Fish. Sci., 68*(3), 509-516.

Baker, D. H. (1986). Problems and pitfalls in animal experiments designed to establish dietary requirements for essential nutrients. *J.Nutr, 116*(12), 2339-2349.

Baker, D. H. (2007). Lysine, arginine, and related amino acids: An introduction to the 6th amino acid assessment workshop. *J. Nutr., 137*(6 Suppl 2), 1599S-1601S. doi:137/6/1599S [pii]

Barman, R. (1991). *A taxonomic revision of the indo-burmese species of danio hamilton buchanan (pisces, cyprinidae)* Zoological Survey of India.

Barnard, D. E., Lewis, S. M., Teter, B. B., & Thigpen, J. E. (2009). Open- and closed-formula laboratory animal diets and their importance to research. *J. Am. Assoc. Lab. Anim. Sci., 48*(6), 709-713.

Berge, G. E., Lied, E., & Sveier, H. (1997). Nutrition of atlantic salmon (salmo salar): The requirement and metabolism of arginine. *Comp. Biochem. Physiol., Part A: Mol. Intergr. Physiol., 117*(4), 501-509.

Berge, G. E., Sveier, H., & Lied, E. (1998). Nutrition of atlantic salmon (salmo salar); the requirement and metabolic effect of lysine. *Comp. Biochem. Physiol., Part A: Mol. Intergr. Physiol., 120*(3), 477-485.

Brunton, J. A., Bertolo, R. F., Pencharz, P. B., & Ball, R. O. (1999). Proline ameliorates arginine deficiency during enteral but not parenteral feeding in neonatal piglets. *Am. J. Physiol., 277*(2 Pt 1), E223-31.

Bureau, D., Kaushik, S., & Cho, C. Y. (2002). Bioenergetics. *Fish Nutr., 3*, 1-59.

Cahu, C. L., Gisbert, E., Villeneuve, L. A., Morais, S., Hamza, N., Wold, P., et al. (2009). Influence of dietary phospholipids on early ontogenesis of fish. *Aquacult. Res., 40*(9), 989-999.

Cao, J. M., Chen, Y., Zhu, X., Huang, Y. H., Zhao, H. X., Li, G. L., . . . Pan, Q. (2012). A study on dietary l-lysine requirement of juvenile yellow catfish pelteobagrus fulvidraco. *Aquacult. Nutr., 18*(1), 35-45. doi:10.1111/j.1365-2095.2011.00874.x

Carpenter, K. J. (1960). The estimation of the available lysine in animal-protein foods. *Biochem. J., 77*, 604-610.

Cho, C. Y., Kaushik, S., & Woodward, B. (1992). Dietary arginine requirement of young rainbow trout (oncorhynchus mykiss). *Comp. Biochem. Physiol., Part A: Mol. Intergr. Physiol., 102*(1), 211-216.

Cho, C., & Kaushik, S. (1990). Nutritional energetics in fish: Energy and protein utilization in rainbow trout (salmo gairdneri). *World Rev. Nutr. Diet., 61*, 132-172.

Cowey, C., & Walton, M. (1988). Studies on the uptake of (14C) amino acids derived from both dietary (14C) protein and dietary (14C) amino acids by rainbow trout, salmo gairdneri richardson. *J. Fish Biol., 33*(2), 293-305.

Cowey, C. B., & Walton, M. J. (1989). Intermediary metabolism. *Fish Nutr., 2*, 259-329.

Dabrowski, K., Zhang, Y., Kwasek, K., Hliwa, P., & Ostaszewska, T. (2010). Effects of protein-, peptide-and free amino acid-based diets in fish nutrition. *Aquacult. Res., 41*(5), 668-683.

Dabrowski, K., Lee, K. J., & Rinchard, J. (2003). The smallest vertebrate, teleost fish, can utilize synthetic dipeptide-based diets. *J. Nutr., 133*(12), 4225-4229.

Darrow, K. O., & Harris, W. A. (2004). Characterization and development of courtship in zebrafish, danio rerio. *Zebrafish, 1*(1), 40-45.

Das, U. N. (2006). Essential fatty acids-a review. *Curr. Pharm. Biotechnol., 7*(6), 467-482.

Dioundick, O., & Stom, D. (1990). Effects of dietary α-cellulose levels on the juvenile tilapia, oreochromis mossambicus (peters). *Aquaculture, 91*(3), 311-315.

Dumas, A., De Lange, C. F., France, J., & Bureau, D. P. (2007). Quantitative description of body composition and rates of nutrient deposition in rainbow trout (oncorhynchus mykiss). *Aquaculture, 273*(1), 165-181.

Fuentes-Appelgren, P., Opazo, R., Barros, L., Feijoó, C. G., Urzúa, V., & Romero, J. (2014). Effect of the dietary inclusion of soybean components on the innate immune system in zebrafish. *Zebrafish, 11*(1), 41-49.

Garling, D.L., and Wilson, R.P. (1976). Optimum dietary protein to energy ratio for channel catfish fingerlings, ictalurus punctatus. *J. Nutr., 106*, 1368.

Gatlin III, D. M. (2002). Nutrition and fish health. *Fish Nutr., 3*, 671-702.

Gerlach, G., & Lysiak, N. (2006). Kin recognition and inbreeding avoidance in zebrafish, danio rerio, is based on phenotype matching. *Anim. Behav., 71*(6), 1371-1377.

Glencross, B. D. (2009). Exploring the nutritional demand for essential fatty acids by aquaculture species. *Rev. Aquac., 1*(2), 71-124.

Glencross, B. D., Boujard, T., & Kaushik, S. J. (2003). Influence of oligosaccharides on the digestibility of lupin meals when fed to rainbow trout, oncorhynchus mykiss. *Aquaculture, 219*(1), 703-713.

Gómez-Requeni, P., Conceicao, L., Jordal, A. O., & Rønnestad, I. (2010). A reference growth curve for nutritional experiments in zebrafish (danio rerio) and changes in whole body proteome during development. *Fish Physiol. Biochem., 36*(4), 1199-1215.

Guillaume, J. (1999). *Nutrition et alimentation des poissons et crustacés* Editions Quae.

Guthrie, D. (1986). Role of vision in fish behaviour. *The behaviour of teleost fishes* (pp. 75-113) Springer.

Halver, J. E. (2002). The vitamins. In J. E. Halver (Ed.), *Fish nutrition* (3rd ed., pp. 61) Academic Press.

Halver, J. E., & Hardy, R. W. (Eds.). (2002). *Fish nutrition* (3rd ed.). San Diego, CA: Academic Press.

Hepher, B. (1988). *Nutrition of pond fishes* Cambridge University Press.

Hilton, J., Atkinson, J., & Slinger, S. (1983). Effect of increased dietary fiber on the growth of rainbow trout (salmo gairdneri). *Can. J. Fish. Aquat. Sci., 40*(1), 81-85.

Horwitz, W. (Ed.). (2006). *Official methods of analysis of the AOAC* (18th ed.). Washington D.C., USA: Association of Official Analytical Chemists.

Hua, K., & Bureau, D. P. (2009). Development of a model to estimate digestible lipid content of salmonid fish feeds. *Aquaculture, 286*(3), 271-276.

Hughes, S. G., Rumsey, G. L., & Nesheim, M. C. (1983). Dietary requirements for essential branched-chain amino acids by lake trout. *Trans. Am. Fish. Soc., 112*(6), 812-817.

Institute of Laboratory Animal Resources (US). Committee on Care, Use of Laboratory Animals, & National Institutes of Health (US). Division of Research Resources. (1985). *Guide for the care and use of laboratory animals* National Academies.

Izquierdo, M., Fernandez-Palacios, H., & Tacon, A. (2001). Effect of broodstock nutrition on reproductive performance of fish. *Aquaculture, 197*(1), 25-42.

Jackson, A., & Capper, B. (1982). Investigations into the requirements of the tilapia sarotherodon mossambicus for dietary methionine, lysine and arginine in semi-synthetic diets. *Aquaculture, 29*(3), 289-297.

Jagadeeswaran, P., & Liu, Y. C. (1997). A hemophilia model in zebrafish: Analysis of hemostasis. *Blood Cells, Mol., Dis., 23*(1), 52-57.

Jaya-Ram, A., Kuah, M. K., Lim, P. S., Kolkovski, S., & Shu-Chien, A. C. (2008). Influence of dietary HUFA levels on reproductive performane, tissue fatty acid profile and desaturase and elongase mRNAs expression in female zebrafish *danio rerio*. *Aquaculture, 277*, 275-281.

Jobling, M. (1995). *Environmental biology of fishes*. London: Chapman and Hall.

Jump, D. B. (2002). Dietary polyunsaturated fatty acids and regulation of gene transcription. *Curr. Opin. Lipidol., 13*(2), 155-164.

Karanth, S., Lall, S. P., Denovan-Wright, E. M., & Wright, J. M. (2009). Differential transcriptional modulation of duplicated fatty acid-binding protein genes by dietary fatty acids in zebrafish (danio rerio): Evidence for subfunctionalization or neofunctionalization of duplicated genes. *BMC Evol. Biol., 9*, 219-2148-9-219. doi:10.1186/1471-2148-9-219 [doi]

Kaushik, S. J. (1998). Nutritional bioenergetics and estimation of waste production in non-salmonids. *Aquat. Living Resour., 11*(04), 211-217.

Kaushik, S. J., & Luquet, P. (1979). Influence of dietary amino acid patterns on the free amino acid contents of blood and muscle of rainbow trout (salmo gairdnerii R.). *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol., 64*(2), 175-180.

Kaushik, S. J., & Seiliez, I. (2010). Protein and amino acid nutrition and metabolism in fish: Current knowledge and future needs. *Aquacult. Res., 41*(3), 322-332.

Kaushik, S., Georga, I., & Koumoundouros, G. (2011). Growth and body composition of zebrafish (danio rerio) larvae fed a compound feed from first feeding onward: Toward implications on nutrient requirements. *Zebrafish, 8*(2), 87-95.

Kaushik, S., & Cowey, C. (1991). Dietary factors affecting nitrogen excretion by fish. *Nutritional Strategies and Aquaculture Waste,* 3-19.

Kaushik, S., Dabrowski, K., & Blanc, D. (1983). Nitrogen and energy utilization in juvenile carp (cyprinus carpio) fed casein, amino acids or a protein-free diet. *Reprod., Nutr., Dev., 23*(4), 741-754.

Kaushik, S., & Fauconneau, B. (1984). Effects of lysine administration on plasma arginine and on some nitrogenous catabolites in rainbow trout. *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol., 79*(3), 459-462.

Kent, M. L., & Varga, Z. (2012). Use of zebrafish in research and importance of health and husbandry. *ILAR J., 53*(2), 89-94. doi:10.1093/ilar.53.2.89 [doi]

Ketola, H. G. (1983). Requirement for dietary lysine and arginine by fry of rainbow trout. *J. Anim. Sci., 56*(1), 101-107.

Kim, K., Grimshaw, T. W., Kayes, T. B., & Amundson, C. H. (1992). Effect of fasting or feeding diets containing different levels of protein or amino acids on the activities of the liver amino acid-degrading enzymes and amino acid oxidation in rainbow trout (oncorhynchus mykiss). *Aquaculture, 107*(1), 89-105.

Kinth, P., Mahesh, G., & Panwar, Y. (2013). Mapping of zebrafish research: A global outlook. *Zebrafish, 10*(4), 510-517.

Klein, R. G., & Halver, J. E. (1970). Nutrition of salmonoid fishes: Arginine and histidine requirements of chinook and coho salmon. *J. Nutr., 100*, 1105-1109.

Krogdahl, Å., HEMRE, G., & Mommsen, T. (2005). Carbohydrates in fish nutrition: Digestion and absorption in postlarval stages. *Aquacult. Nutr., 11*(2), 103-122.

Kwasek, K., Dabrowski, K., Ware, K., Reddish, J. M., & Wick, M. (2012). The effect of lysine‐supplemented wheat gluten‐based diet on yellow perch perca flavescens (mitchill) performance. *Aquacult. Res., 43*(9), 1384-1391.

Kwasek, K., Dabrowski, K., Nynca, J., Takata, R., Wojno, M., & Wick, M. (2014). The influence of dietary lysine on yellow perch female reproductive performance and the quality of eggs. *N. Am. J. Aquacult., 76*(4), 351-358.

Laale, H. W. (1977). The biology and use of zebrafish, brachydanio rerio in fisheries research. *J. Fish Biol., 10*(2), 121-173.

Lall, S. P. (2002). The minerals. In J. E. Halver (Ed.), *Fish nutrition* (3rd ed., pp. 259) Academic Press.

Langheinrich, U. (2003). Zebrafish: A new model on the pharmaceutical catwalk. *BioEssays, 25*(9), 904-912.

Lawrence, C. (2007). The husbandry of zebrafish (*danio rerio*): A review. *Aquaculture, 269*, 1.

Lawrence, C. (2011). Advances in zebrafish husbandry and management. *Methods Cell Biol., 104*, 431-451.

Limsuwan, T., & Lovell, R. T. (1981). Intestinal synthesis and absorption of vitamin B-12 in channel catfish. *J.Nutr, 101*, 2125-2132.

Lin, Y., Gong, Y., Yuan, Y., Gong, S., Yu, D., & Li, Q. (2013). Dietary L-lysine requirement of juvenile chinese sucker, *myxocyprinus asiaticus*. *Aquacult. Res., 44*, 1539.

Liqing, Z., Shimada, Y., Nishimura, Y., Tanaka, T., & Nishimura, N. (2013). A novel, reliable method for repeated blood collection from aquarium fish. *Zebrafish, 10*(3), 425.

Luzzana, U., Hardy, R. W., & Halver, J. E. (1998). Dietary arginine requirement of fingerling coho salmon (oncorhynchus kisutch). *Aquaculture, 163*(1), 137-150.

Mambrini, M., & Kaushik, S. (1995). Indispensable amino acid requirements of fish: Correspondence between quantitative data and amino acid profiles of tissue proteins. *J. Appl. Ichthyol., 11*(3‐4), 240-247.

Markovich, M. L., Rizzuto, N. V., & Brown, P. B. (2007). Diet affects spawning in zebrafish. *Zebrafish, 4*(1), 69-74.

McClure, M., McIntyre, P., & McCune, A. (2006). Notes on the natural diet and habitat of eight danionin fishes, including the zebrafish danio rerio. *J. Fish Biol., 69*(2), 553-570.

McLean, E., Rønsholdt, B., & Sten, C. (1999). Gastrointestinal delivery of peptide and protein drugs to aquacultured teleosts. *Aquaculture, 177*(1), 231-247.

Mehrad, B., Jafaryan, H., & Taati, M. M. (2011). Impact of different dietary vitamin C contents on growth, survival, fecundity and egg diameter in the zebrafish, danio rerio (pisces, cyprinidae). *Anim. Biol. Anim. Husb., 3*(1), 18-25.

Meinelt, B. T., Schulz, C., Wirth, M., Kuerzinger, H., & Steinberg, C. (1999). Dietary fatty acid composition influences the fertilization rate of zebrafish (danio rerio Hamilton‐Buchanan). *J. Appl. Ichthyol., 15*(1), 19-23.

Meinelt, T., Schulz, C., Wirth, M., Kürzinger, H., & Steinberg, C. (2000). Correlation of diets high in n-6 polyunsaturated fatty acids with high growth rate in zebrafish (danio rerio). *Comp. Med., 50*(1), 43-45.

Miller, G. W., Labut, E. M., Lebold, K. M., Floeter, A., Tanguay, R. L., & Traber, M. G. (2012). Zebrafish (danio rerio) fed vitamin E-deficient diets produce embryos with increased morphologic abnormalities and mortality. *J. Nutr. Biochem., 23*(5), 478-486.

Moon, T. W. (2001). Glucose intolerance in teleost fish: Fact or fiction? *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol., 129*(2), 243-249.

Moughan, P. J., & Rutherfurd, S. M. (1996). A new method for determining digestible reactive lysine in foods. *J. Agric. Food Chem., 44*(8), 2202-2209.

Murai, T., Akiyama, T., & Nose, T. (1981). Use of crystalline amino acids coated with casein in diets for carp. *Bull. Jap. Soc. Sci. Fish., 47*(4), 523-527.

Murai, T., Ogata, H., Hirasawa, Y., Akiyama, T., & Nose, T. (1987). Portal absorption and hepatic uptake of amino acids in rainbow trout [onchorhynchus masou] force-fed complete diets containing casein or crystalline amino acids. *Bull. Jap. Soc. Sci. Fish.*

National Research Council. Committee on the Nutrient Requirements of Fish and Shrimp. (2011). *Nutrient requirements of fish and shrimp* National academies press.

Nose, T. (1979). Summary report on the requirements of essential amino acids for carp. In J. Halver (Ed.), *Finfish nutrition and fishfeed technology* (1st ed., pp. 145)

Nose, T., Arai, S., Lee, D., & Hashimoto, Y. (1974). A note on amino acids essential for growth of young carp. *Bull. Jap. Soc. Sci. Fish.*

O'Brine, T. M., Vrtělová, J., Snellgrove, D. L., Davies, S. J., & Sloman, K. A. (2015). Growth, oxygen consumption, and behavioral responses of danio rerio to variation in dietary protein and lipid levels. *Zebrafish, 12*(4), 296-304.

Padgett-Vasquez, S., Siccardi III, A., D'Abramo, L., & Watts, S. (2008). The effect of dietary calcium on zebrafish (danio rerio), growth, survival, and bone mineral density (BMD). Paper presented at the *85th Annual Meeting of the Alabama Academy of Science, Birmingham, AL,*

Pellett, P. L., & Young, V. R. (1984). Evaluation of the use of amino acid composition data in assessing the protein quality of meat and poultry products. *Am. J. Clin. Nutr., 40*(3 Suppl), 718-736.

Penglase, S., Moren, M., & Hamre, K. (2012). Lab animals: Standardize the diet for zebrafish model. *Nature, 491*(7424), 333-333.

Quintero, H., Durland, E., ALLEN DAVIS, D., & Dunham, R. (2011). Effect of lipid supplementation on reproductive performance of female channel catfish, ictalurus punctatus, induced and strip‐spawned for hybridization. *Aquacult. Nutr., 17*(2), 117-129.

Ramsay, J. M., Feist, G. W., Varga, Z. M., Westerfield, M., Kent, M. L., & Schreck, C. B. (2006). Whole-body cortisol is an indicator of crowding stress in adult zebrafish, danio rerio. *Aquaculture, 258*(1), 565-574.

Ren, M., Liao, Y., Xie, J., Liu, B., Zhou, Q., Ge, X., et al. (2013). Dietary arginine requirement of juvenile blunt snout bream, megalobrama amblycephala. *Aquaculture, 414*, 229-234.

Robinson, E. H., Wilson, R. P., & Poe, W. E. (1981). Arginine requirement and apparent absence of a lysine-arginine antagonist in fingerling channel catfish. *J. Nutr., 111*, 46.

Robinson, E. H., Wilson, R. P., & Poe, W. E. (1980). Re-evaluation of the lysine requirement and lysine utilization by fingerling channel catfish. *J. Nutr., 110*(11), 2313-2316.

Robison, B. D., Drew, R. E., Murdoch, G. K., Powell, M., Rodnick, K. J., Settles, M., et al. (2008). Sexual dimorphism in hepatic gene expression and the response to dietary carbohydrate manipulation in the zebrafish (danio rerio). *Comp. Biochem. Physiol., Part D: Genomics Proteomics, 3*(2), 141-154.

Sampath, H., & Ntambi, J. M. (2005). Polyunsaturated fatty acid regulation of genes of lipid metabolism. *Annu. Rev. Nutr., 25*, 317-340.

Santiago, C. B., & Lovell, R. T. (1988). Amino acid requirements for growth of nile tilapia. *J. Nutr.,* 1540-1546.

Santoriello, C., & Zon, L. I. (2012). Hooked! modeling human disease in zebrafish. *J. Clin. Invest., 122*(7), 2337.

Sargent, J., Bell, J., Bell, M., Henderson, R., & Tocher, D. (1995). Requirement criteria for essential fatty acids. *J. Appl. Ichthyol., 11*(3‐4), 183-198.

Sassi, M. (2001). *Carboxyterminal degradation products of type I collagen* Oulun yliopisto.

Schwerte, T., Voigt, S., & Pelster, B. (2005). Epigenetic variations in early cardiovascular performance and hematopoiesis can be explained by maternal and clutch effects in developing zebrafish (danio rerio). *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol., 141*(2), 200-209.

Segovia-Quintero, M. A., & Reigh, R. C. (2004). Coating crystalline methionine with tripalmitin-polyvinyl alcohol slows its absorption in the intestine of nile tilapia, oreochromis niloticus. *Aquaculture, 238*(1), 355-367.

Shearer, K. D. (1994). Factors affecting the proximate composition of cultured fishes with emphasis on salmonids. *Aquaculture, 119*(1), 63-88.

Shearer, K. D., & Swanson, P. (2000). The effect of whole body lipid on early sexual maturation of 1 age male chinook salmon (oncorhynchus tshawytscha). *Aquaculture, 190*(3), 343-367.

Shiau, S., Yu, H., Hwa, S., Chen, S., & Hsu, S. (1988). The influence of carboxymethylcellulose on growth, digestion, gastric emptying time and body composition of tilapia. *Aquaculture, 70*(4), 345-354.

Shi-Yen, S., & Chun-Qui, L. (1993). No dietary vitamin B 12 required for juvenile tilapia oreochromis niloticus× O. aureus. *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol., 105*(1), 147-150.

Siccardi III, A. J., Garris, H. W., Jones, W. T., Moseley, D. B., D'Abramo, L. R., & Watts, S. A. (2009). Growth and survival of zebrafish (danio rerio) fed different commercial and laboratory diets. *Zebrafish, 6*(3), 275-280.

Smith Jr, D. L., Barry, R. J., Powell, M. L., Nagy, T. R., D'Abramo, L., & Watts, S. A. (2013). Dietary protein source influence on body size and composition in growing zebrafish. *Zebrafish, 10*(3), 439-446.

Spence, R., Fatema, M., Ellis, S., Ahmed, Z., & Smith, C. (2007). Diet, growth and recruitment of wild zebrafish in bangladesh. *J. Fish Biol., 71*(1), 304-309.

Spence, R., Fatema, M., Reichard, M., Huq, K., Wahab, M., Ahmed, Z., et al. (2006). The distribution and habitat preferences of the zebrafish in bangladesh. *J. Fish Biol., 69*(5), 1435-1448.

Spence, R., Gerlach, G., Lawrence, C., & Smith, C. (2008). The behaviour and ecology of the zebrafish, danio rerio. *Biol. Rev., 83*(1), 13-34.

Stoletov, K., Fang, L., Choi, S. H., Hartvigsen, K., Hansen, L. F., Hall, C., et al. (2009). Vascular lipid accumulation, lipoprotein oxidation, and macrophage lipid uptake in hypercholesterolemic zebrafish. *Circ. Res., 104*(8), 952-960. doi:10.1161/CIRCRESAHA.108.189803 [doi]

Stone, D., Allan, G., & Anderson, A. (2003). Carbohydrate utilization by juvenile silver perch, bidyanus bidyanus (mitchell). II. digestibility and utilization of starch and its breakdown products. *Aquacult. Res., 34*(2), 109-121.

Suarez, R. K., & Mommsen, T. P. (1987). Gluconeogenesis in teleost fishes. *Can. J. Zool., 65*(8), 1869-1882.

Tacon, A. (1996). Lipid nutritional pathology in farmed fish. *Arch. Anim. Nutr., 49*(1), 33-39.

Takeuchi, T., Arai, S., Watanabe, T., & Shimma, Y. (1980). Requirement of eel anguilla japonica for essential fatty acids. *Bull. Jpn. Soc. Sci. Fish., 46*(3), 345-353.

Talwar, P., & Jhingaran, A. (1991) Inland fishes of india and adjacent countries. CRC Press.

Tandler, A., Harel, M., Koven, W., & Kolkovsky, S. (1995). Broodstock and larvae nutrition in gilthead seabream sparus aurata new findings on its involvement in improving growth, survival and swim bladder inflation. *Isr. J. Aquacult. Bamidgeh., 47*, 95-111.

Tantikitti, C., & March, B. (1995). Dynamics of plasma free amino acids in rainbow trout (oncorhynchus mykiss under variety of dietary conditions. *Fish Physiol. Biochem., 14*(3), 179-194.

Teshima, S., Kanazawa, A., & Koshio, S. (1990). Effects of methionine-enriched plastein supplemented to soybean-protein based diets on common carp cyprinus carpio and tilapia oreochromis niloticus. Paper presented at the *The Second Asian Fisheries Forum,* pp. 279-282.

Tibaldi, E., Tulli, F., & Lanari, D. (1994). Arginine requirement and effect of different dietary arginine and lysine levels for fingerling sea bass (dicentrarchus labrax). *Aquaculture, 127*(2), 207-218.

Tocher, D. R., Bendiksen, E. Å., Campbell, P. J., & Bell, J. G. (2008). The role of phospholipids in nutrition and metabolism of teleost fish. *Aquaculture, 280*(1), 21-34.

Tomlinson, C., Rafii, M., Ball, R. O., & Pencharz, P. B. (2011). Arginine can be synthesized from enteral proline in healthy adult humans. *J. Nutr., 141*(8), 1432-1436. doi:10.3945/jn.110.137224 [doi]

Toth, E. (1986). Arginine requirement of wels (silurus glanis L.). *Aquacult. Hung., 5*, 59-63.

Twibell, R., Griffin, M., Martin, B., Price, J., & Brown, P. (2003). Predicting dietary essential amino acid requirements for hybrid striped bass. *Aquacult. Nutr., 9*(6), 373-381.

Ulloa, P. E., Iturra, P., Neira, R., & Araneda, C. (2011). Zebrafish as a model organism for nutrition and growth: Towards comparative studies of nutritional genomics applied to aquacultured fishes. *Rev. Fish Biol. Fisheries, 21*(4), 649-666.

Van den Hurk, R., & Lambert, J. (1983). Ovarian steroid glucuronides function as sex pheromones for male zebrafish, brachydanio rerio. *Can. J. Zool., 61*(11), 2381-2387.

Van den Hurk, R., Schoonen, W., Van Zoelen, G., & Lambert, J. (1987). The biosynthesis of steroid glucuronides in the testis of the zebrafish, brachydanio rerio, and their pheromonal function as ovulation inducers. *Gen. Comp. Endocrinol., 68*(2), 179-188.

Walton, M. J., & Cowey, C. B. (1982). Aspects of intermediary metabolism in salmonid fish. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol., 73*(1), 59-79.

Walton, M., Cowey, C., Coloso, R. M., & Adron, J. (1986). Dietary requirements of rainbow trout for tryptophan, lysine and arginine determined by growth and biochemical measurements. *Fish Physiol. Biochem., 2*(1-4), 161-169.

Wan, J., Mai, K., & Ai, Q. (2006). The recent advance on arginine nutritional physiology in fish. *J. Fish. Sci. China, 13*, 679-685.

Wang, S., Liu, Y., Tian, L., Xie, M., Yang, H., Wang, Y., et al. (2005). Quantitative dietary lysine requirement of juvenile grass carp ctenopharyngodon idella. *Aquaculture, 249*(1), 419-429.

Watanabe, T., Satoh, S., & Takeuchi, T. (1988). Availability of minerals in fish meal to fish. *Asian Fish. Sci., 1*(2), 75-195.

Watanabe, T. (1985). Importance of the study of broodstock nutrition for further development of aquaculture. *Nutrition and Feeding in Fish.* Academic Press, London.

Watts, S. A., Powell, M., & D'Abramo, L. R. (2012). Fundamental approaches to the study of zebrafish nutrition. *ILAR J., 53*(2), 144-160. doi:10.1093/ilar.53.2.144 [doi]

Wilson, R. P., Harding, D. E., & Garling, D. L. (1977). Effect of dietary pH on amino acid utilization and the lysine requirement of fingerling channel catfish. *J. Nutr., 107*, 166.

Wilson, R. P. (2002). Amino acids and proteins. In Halver, J. E., & Hardy, R. W. (Eds.), *Fish Nutrition* (143-179). San Diego, CA: Academic Press.

Wilson, R. P., & Poe, W. E. (1987). Apparent inability of channel catfish to utilize dietary mono- and disaccharides as energy sources. *J. Nutr., 117*(2), 280-285.

Yamada, S., Simpson, K., & Tanaka, T. (1981). Plasma amino acid changes in rainbow trout salmo gairdneri force-fed casein and a corresponding amino acid mixture. *Bull. Jpn. Soc. Sci. Fish., 47*(8), 1035-1040.

Zarate, D. D., & Lovell, R. T. (1997). Free lysine (L-lysine· HCl) is utilized for growth less efficiently than protein-bound lysine (soybean meal) in practical diets by young channel catfish (ictalurus punctatus). *Aquaculture, 159*(1), 87-100.

Zarate, D., Lovell, R., & Payne, D. (1999). Effects of feeding frequency and rate of stomach evacuation on utilization of dietary free and protein-bound lysine for growth by channel catfish ictalurus punctatus. *Aquacult. Nutr., 5*(1), 17-22.

Zheng, D., Kille, P., Feeney, G. P., Cunningham, P., Handy, R. D., & Hogstrand, C. (2010). Dynamic transcriptomic profiles of zebrafish gills in response to zinc supplementation. *BMC Genomics,* 11(1), 553.

Zhou, X., Zhao, C., & Lin, Y. (2007). Compare the effect of diet supplementation with uncoated or coated lysine on juvenile jian carp (cyprinus carpio var. jian). *Aquacult. Nutr., 13*(6), 457-461.

Zhou, F., Shao, J., Xu, R., Ma, J., & Xu, Z. (2010). Quantitative l-lysine requirement of juvenile black sea bream (sparus macrocephalus). *Aquacult. Nutr. 16*(2), 194-204.

APPENDIX

Table A1. Mean plasma amino acid concentrations* of fish fed diets of varying arginine concentrations in Experiment 2.

	Amino Acids (uM)			
Arginine Level	**Arginine**	**Citrulline**	**Ornithine**	**Lysine**
0.80	92.1 ± 9.1	25.2 ± 7.3[a]	12.3 ± 2.3	183.3 ± 49.5
1.08	85.8 ± 16.5	18.6 ± 6.2[ab]	11.1 ± 1.5	154.0 ± 16.7
1.43	148.7 ±67.1	20.2 ± 0.5[ab]	27.0 .± 16.4	302.7 ± 131.7
1.56	98.5 ± 61.1	11.3 ± 0.9[b]	19.3 ± 10.1	173.4 ± 59.5
1.88	91.5 ± 11.3	15.9 ± 0.8[ab]	13.7 ± 1.4	169.6 ± 27.0
2.37	94.2 ± 28.4	15.4 ±4.1[ab]	16.8 ± 3.1	210.7 ± 62.3
2.70	97.2 ± 44.1	18.6 ± 2.6[ab]	12.1 ± 3.1	207.3 ± 90.2

*Amino acid concentration values are means of triplicate groups ± SD.
[a,b] Means within a column with uncommon superscripts differ ($P < 0.05$).

Table A1. (continued).

	Amino Acids (uM)			
Arginine Level	**Histidine**	**Alanine**	**Glycine**	**Taurine**
0.80	317.5 ± 83.1^{ab}	232.4 ± 27.9	313.8 ± 33.0	1713.8 ± 63.8^{ab}
1.08	223.7 ± 17.9^{a}	199.3 ± 35.1	269.5 ± 8.8	958.6 ± 95.6^{a}
1.43	282.7 ± 17.9^{ab}	237.5 ± 34.5	334.6 ± 17.8	1184.9 ± 273.2^{ab}
1.56	235.6 ± 16.5^{a}	183.3 ± 55.0	298.6 ± 121.1	1161.5 ± 422.5^{ab}
1.88	444.0 ± 136.9^{b}	238.0 ± 47.5	376.2 ± 25.1	1815.0 ± 302.3^{b}
2.37	272.3 ± 7.8^{ab}	217.1 ± 29.7	332.3 ± 48.1	1353.1 ± 362.1^{ab}
2.70	392.8 ± 68.6^{ab}	220.8 ± 27.8	308.5 ± 42.8	1830.4 ± 315.2^{b}

*Amino acid concentration values are means of triplicate groups ± SD.
[a,b] Means within a column with uncommon superscripts differ ($P < 0.05$).

Table A1. (continued).

	Amino Acids (uM)			
Arginine Level	**Methionine**	**Glutamine**	**Glutamate**	**Asparagine**
0.80	51.5 ± 13.8	249.6 ±17.6	380.6 ± 142.8	38.2 ± 33.3
1.08	48.4 ± 4.9	276.4 ± 22.2	177.9 ± 41.2	49.3 ± 27.5
1.43	64.2 ± 12.5	331.1 ± 15.2	229.6 ± 92.5	102.3 ± 9.6
1.56	48.5 ± 11.4	280.1 ± 80.8	179.2 ± 11.2	75.9 ± 15.1
1.88	51.1 ± 8.0	377.3 ± 31.4	390.9 ± 92.6	99.5 ± 50.2
2.37	41.6 ± 4.6	371.1 ± 67.6	186.2 ± 36.5	114.7 ± 20.1
2.70	43.4 ± 7.5	284.3 ± 82.0	289.2 ± 73.5	87.6 ± 22.4

*Amino acid concentration values are means of triplicate groups ± SD.
[a,b] Means within a column with uncommon superscripts differ ($P < 0.05$).

Table A1. (continued).

	Amino Acids (uM)			
Arginine Level	**Aspartic acid**	**Serine**	**Threonine**	**Valine**
0.80	197.9 ± 72.2	197.4 ± 36.2	209.5 ± 17.0	229.8 ± 31.7
1.08	98.4 ± 54.9	163.7 ± 18.9	255.2 ± 35.3	234.9 ± 12.9
1.43	111.5 ± 62.9	231.4 ± 27.2	269.4 ± 37.3	263.5 ± 42.1
1.56	114.1 ± 12.6	176.2 ± 28.1	198.2 ± 36.4	210.9 ± 58.0
1.88	173.0 ± 26.6	216.5 ± 36.8	294.8 ± 70.8	226.6 ± 29.4
2.37	73.1 ± 38.7	174.9 ± 14.9	288.6 ± 43.8	252.1 ± 78.3
2.70	132.2 ± 83.8	193.1 ± 27.5	212.8 ± 18.5	211.7 ± 14.4

*Amino acid concentration values are means of triplicate groups ± SD.
[a,b] Means within a column with uncommon superscripts differ ($P < 0.05$).

Table A1. (continued).

	Amino Acids (uM)			
Arginine Level	**Isoleucine**	**Leucine**	**Proline**	**Tyrosine**
0.80	110.0 ± 11.5	178.4 ± 23.8	640.2 ± 102.7	65.0 ± 28.8
1.08	103.3 ± 6.8	165.7 ± 12.3	799.0 ± 290.5	54.0 ± 17.6
1.43	115.3 ± 19.1	180.7 ± 37.2	799.7 ± 230.6	70.3 ± 12.3
1.56	96.0 ± 29.9	144.7 ± 50.0	778.3 ± 103.5	54.2 ± 22.8
1.88	102.0 ± 11.1	157.2 ± 20.4	907.7 ± 160.6	60.7 ± 34.7
2.37	115.1 ± 49.5	179.6 ± 73.5	960.5 ± 152.2	58.9 ± 27.1
2.70	99.9 ± 8.8	147.3 ± 13.2	616.6 ± 195.4	55.7 ± 23.0

*Amino acid concentration values are means of triplicate groups ± SD.
[a,b] Means within a column with uncommon superscripts differ ($P < 0.05$).

Table A1. (continued).

	Amino Acids (uM)		
Arginine Level	**Phenylalanine**	**Tryptophan**	**Total AAs**
0.80	97.7 ± 17.5	24.0 ± 1.6	5560.2 ± 374.4
1.08	152.5 ± 69.0	30.2 ± 3.4	4529.7 ± 247.4
1.43	147.4 ± 36.6	32.3 ± 3.5	5486.8 ± 983.4
1.56	141.9 ± 72.5	27.0 ± 10.6	4706.4 ± 1117.6
1.88	91.8 ± 7.4	23.2 ± 4.8	6336.1 ± 944.6
2.37	174.1 ± 124.8	29.4 ± 6.0	5531.8 ± 734.0
2.70	106.7 ± 11.8	26.5 ± 0.9	5594.8 ± 1015.8

*Amino acid concentration values are means of triplicate groups ± SD.
[a,b] Means within a column with uncommon superscripts differ ($P < 0.05$).